PATENT

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PATENT APPLICATION

for

TETRAPROPYLAMMONIUM TETRATHIOMOLYBDATE AND RELATED COMPOUNDS FOR ANTI-ANGIOGENIC THERAPIES

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BACKGROUND OF THE INVENTION

The present application claims priority to co-pending U.S. provisional application Serial No. 60/397,804, filed July 23, 2002, the entire disclosure of which is incorporated herein by reference without disclaimer.

1. Field of the Invention

The present invention relates generally to the field of angiogenic diseases. More particularly, it provides copper binding compounds with improved properties and methods of using such compounds in the prevention and treatment of diseases with an angiogenic component, including as cancer. Pharmaceutical compositions, therapeutic kits and combination treatment methods are also provided.

2. Description of Related Art

Solid tumors require blood vessel proliferation (angiogenesis) for sustained growth in order to maintain adequate nutrition to other than the most peripheral cell layers (Hayes, 1994; Horak *et al.*, 1993; Parangi *et al.*, 1996). Normal adult human tissues, on the other hand, require little new blood vessel growth, except for wound repair, regeneration following trauma or surgery, and proliferation of the inner lining of the uterus during the menstrual cycle. Thus, dependency on angiogenesis is a fundamental difference between tumor and normal tissue. This difference is quantitatively more striking than the differences in cell replication and cell death rates, on which many cytoreductive chemotherapies depend. As a result of tumor dependency on angiogenesis, the concept of anti-angiogenic therapy for malignancies was developed (Folkman, 1995a; Folkman, 1995b; Hanahan and Folkman, 1996).

There are numerous other examples of diseases characterized by aberrant angiogenesis. One example of such a disease mediated by angiogenesis is ocular neovascular disease. This disease is characterized by invasion of new blood vessels into the structures of the eye such as the retina or cornea. It is the most common cause of blindness and is

involved in approximately twenty eye diseases. In age-related macular degeneration, the associated visual problems are caused by an ingrowth of chorioidal capillaries through defects in Bruch's membrane with proliferation of fibrovascular tissue beneath the retinal pigment epithelium.

Another disease in which angiogenesis is believed to be involved is rheumatoid arthritis. The blood vessels in the synovial lining of the joints undergo angiogenesis. In addition to forming new vascular networks, the endothelial cells release factors and reactive oxygen species that lead to pannus growth and cartilage destruction. The factors involved in angiogenesis may actively contribute to, and help maintain, the chronically inflamed state of rheumatoid arthritis.

Copper is both a requirement and a potent stimulus for angiogenesis, as shown by studies of neovascularization in the rabbit cornea (Parke *et al.*, 1988). During prostaglandin E1 (PGE1)-induced angiogenesis in the rabbit cornea, copper accumulates at the site where angiogenesis occurs (Parke *et al.*, 1988). Conversely, in copper deficient rabbits, angiogenesis in the rabbit cornea in response to PGE1 is greatly reduced. In the rabbit cornea, copper for angiogenesis can be supplied by ceruloplasmin (a copper protein) as well as by dissolved copper sulfate, while apoceruloplasmin (ceruloplasmin without copper) does not support angiogenesis (Gullino, 1986). Additional studies have also shown that copper is an important angiogenic agent (Raju *et al.*, 1982; Ziche *et al.*, 1982). These studies all support the concept that unbound copper is required for angiogenesis.

Several years ago, some animal tumor model studies were carried out using an anti-copper approach (Brem *et al.*, 1990a; 1990b; Yoshida *et al.*, 1995). The chelator penicillamine plus a low-copper diet were used to lower copper levels in rats and rabbits with implanted intracerebral tumors. However, the animals treated with the low-copper regimen, while showing reduction in tumor size, did not show improved survival over untreated controls.

Penicillamine therapy has also been reported to be associated with significant side effects, including nausea and abdominal discomfort, and more serious side effects such as

leukopenia and thrombocytopenia, which can lead to aplastic anemia. Nephrotic syndrome has also been reported in certain instances.

The negative reports in the literature, including the Brem *et al.* (1990b) study in which death in the treated animals occurred at the same rate as in untreated control animals, largely discouraged further work in this area. However, in overcoming such prejudices, successful anti-angiogenic therapies were ultimately developed based upon effective modulation of total-body copper status (PCT Application WO 00/13712). The basis of this work involved the determination of a window of copper deficiency, within which angiogenesis can be inhibited, but necessary copper-dependent cellular processes are maintained sufficiently to avoid toxicity. Effective therapy within this "window" was achieved using a range of agents that bind copper and form agent-copper-protein complexes, such as tetrathiomolybdate (TM), and dramatic successes were reported in clinical trials (PCT Application WO 00/13712).

However, despite these advances, there remains in the art a need for improved agents for use in anti-angiogenic therapy via copper reduction and maintenance. The development of compounds with improved stability and shelf life is particularly desirable. In trying to develop more stable copper binding compounds, it would be important to overcome problems typically encountered in such pursuits, such as lower solubility and/or reductions in therapeutic activity. Accordingly, the ability to prepare pharmaceutical formulations of soluble and therapeutically effective copper-binding compounds with improved stability and shelf life would represent a particularly significant advance.

SUMMARY OF THE INVENTION

The present invention solves such needs in the art by providing a range of compounds that bind copper and form tripartite compound-copper-protein complexes, which compounds exhibit increased stability without significant loss of solubility or therapeutic efficacy. The compounds, pharmaceutical formulations and kits of the invention therefore have various advantages in anti-angiogenic and anti-tumor therapies in the clinic, including the ease of preparation and handling, and the increased shelf life, which benefits are achieved without limiting the therapeutic profile of the compounds. The invention provides the underlying compounds, pharmaceuticals, medicaments and kits, as well as the preventative and

therapeutic methods for use in safe and effective intervention in angiogenic diseases, including cancer.

The invention particularly provides compounds, compositions, pharmaceutical formulations and kits comprising a biologically or therapeutically effective amount of at least a first alkylammonium thiomolybdate compound, and related treatment methods and medical uses thereof. The compositions are preferably pharmaceutical compositions or formulations, which comprise a pharmaceutically acceptable excipient along with the at least a first alkylammonium thiomolybdate compound.

As used throughout the entire application, the terms "a" and "an" are used in the sense that they mean "at least one", "at least a first", "one or more" or "a plurality" of the referenced components or steps, except in instances wherein an upper limit is thereafter specifically stated or would be clearly understood by those of ordinary skill in the art. Therefore, "an alkylammonium thiomolybdate compound" means "at least a first alkylammonium thiomolybdate compound". The operable limits and parameters of combinations, as with the amounts of any single agent, will be known to those of ordinary skill in the art in light of the present disclosure.

The "a" and "an" terms are also used to mean "at least one", "at least a first", "one or more" or "a plurality" of steps in the recited methods, except where specifically stated. This is particularly relevant to the administration steps in the treatment methods. Thus, not only may different doses be employed with the present invention, but different numbers of doses may be used, up to and including multiple administrations.

Throughout the present specification and claims, the term "or" is used in the sense that it means "and/or" in reference to the disclosed and claimed components and steps, except in instances wherein a different meaning is thereafter specifically stated or would be clearly understood by one of ordinary skill in the art. Therefore, the term "monoalkylammonium, dialkylammonium, trialkylammonium or tetraalkylammonium", as used herein, means "monoalkylammonium, dialkylammonium, trialkylammonium or tetraalkylammonium" as well as combinations thereof, such as "monoalkylammonium and dialkylammonium; monoalkylammonium and tetraalkylammonium; dialkylammonium and trialkylammonium";

and such like. Thus, unless otherwise expressly stated or clearly known by those of ordinary skill in the art, the term "or" is simply used as a succinct reference term to cover each recited component or step and all combinations thereof.

The compositions, pharmaceutical compositions, medicaments and kits of the present invention comprise more stable agents that still lower copper levels by forming a "tripartite agent-copper-protein complex" that is subsequently cleared from the body. The copper bound in these "tripartite agent-copper-protein complexes" is not reversibly released from these complexes, which distinguishes the invention from reversible bipartite copper chelation.

In certain embodiments, the compositions, pharmaceutical compositions, medicaments and kits of the invention comprise a pharmaceutically acceptable excipient and at least a first alkylammonium thiomolybdate compound that is substantially stable upon exposure to air and moisture, retains solubility and that releases a biologically or therapeutically effective thiomolybdate compound in solution.

The number of alkyl groups in the alkylammonium thiomolybdate compounds can be varied in the practice of the invention. Exemplary embodiments of the invention thus include monoalkylammonium, dialkylammonium, trialkylammonium and tetraalkylammonium thiodimolybdate compounds, and pharmaceutical compositions thereof. Tetraalkylammonium thiodimolybdate compounds will be preferred in certain embodiments.

The number of sulfur groups can also be varied, as known in thiomolybdate chemistry. Further examples of compounds of the invention therefore include alkylammonium monothiomolybdate, dithiomolybdate, trithiomolybdate, tetrathiomolybdate, octathiomolybdate and dodecathiodimolybdate compounds, and pharmaceutical compositions thereof.

Alkylammonium thiomolybdate compounds and pharmaceutical compositions of the invention further include those in which the thiomolybdate compound comprises at least a first iron atom or at least a first oxygen atom. One particular example is alkylammonium iron

octathiodimolybdate. It will be understood that complete oxidation of the thiomolybdate compound should be avoided.

In certain preferred embodiments, the invention provides compositions, pharmaceutical compositions, medicaments and kits comprising a pharmaceutically acceptable excipient and at least a first alkylammonium tetrathiomolybdate compound that comprises a number of alkyl groups sufficient to protect the tetrathiomolybdate from oxidation upon exposure to air and moisture, thereby increasing the stability of the tetrathiomolybdate compound, wherein the alkylammonium tetrathiomolybdate compound releases a biologically or therapeutically effective amount of tetrathiomolybdate in solution.

In other preferred embodiments, the invention provides tetraalkylammonium tetrathiomolybdate compounds and pharmaceutical compositions thereof. The nature of the alkyl groups is not limiting on the invention, which therefore includes tetramethylammonium tetrathiomolybdate, tetraethylammonium tetrathiomolybdate and tetrabutylammonium tetrathiomolybdate and pharmaceutical compositions thereof. Tetrapropylammonium tetrathiomolybdate and pharmaceutical compositions thereof will be preferred in certain aspects of the invention.

Such compositions, pharmaceutical compositions, medicaments and kits thus comprise a pharmaceutically acceptable excipient and at least a first tetraalkylammonium tetrathiomolybdate compound in which the alkyl groups protect the tetrathiomolybdate from oxidation upon exposure to air and moisture, thereby increasing the stability of the tetrathiomolybdate compound, wherein the tetraalkylammonium tetrathiomolybdate compound retains solubility and releases substantially biologically or therapeutically active tetrathiomolybdate and substantially biologically inert alkylammonium groups in aqueous solution.

The invention also provides compositions, pharmaceutical compositions, medicaments and kits that comprise a biologically or therapeutically effective amount of a tetraalkylammonium tetrathiomolybdate compound a pharmaceutically acceptable excipient; wherein the tetraalkylammonium tetrathiomolybdate compound is substantially stable in moist heated air for at least about 7 days; has a half life when exposed to air at room

temperature of at least twice that of ammonium tetrathiomolybdate; is soluble to at least about 1mg/ml in water; and in aqueous solution releases tetrathiomolybdate with substantially intact copper binding properties.

Certain preferred compositions and pharmaceutical compositions that provide such advantageous properties are those in which the at least a first tetraalkylammonium tetrathiomolybdate compound is tetrapropylammonium tetrathiomolybdate. Compositions, medicaments and kits comprising a biologically effective amount of tetrapropylammonium tetrathiomolybdate and a pharmaceutically acceptable excipient are thus preferred embodiments of the invention.

The nature of the pharmaceutical compositions and pharmaceutically acceptable excipients is not critical to the practice of the invention. Typically, such compositions and excipients will be selected to match the angiogenic condition to be treated, such as being formulated for intravenous administration, oral administration, ophthalmic administration and such like. An advantage of the invention is that the systemic administration of the compositions and compounds is effective to treat a wide variety of conditions. Compositions formulated for oral administration are particularly preferred. Nonetheless, the local administration to particular sites is also contemplated, hence the ophthalmic, topical and other formulations of the invention.

Any given composition or pharmaceutical composition of the invention may comprise one or more of the alkylammonium thiomolybdate compounds, such as comprising at least two, three, four or more of such compounds. Compositions and pharmaceutical compositions comprising a plurality of alkylammonium thiomolybdate compounds are also included within the invention.

The compositions and pharmaceutical compositions of the invention may also comprise biologically or therapeutically effective amounts of additional biological and/or therapeutic agents, such as comprising a biologically or therapeutically effective amount of at least a second, third, fourth or further biological and/or therapeutic agent. Naturally, the language "at least a second biological and/or therapeutic agent" is chosen in reference to the

"at least a first alkylammonium thiomolybdate compound" being the first biological and therapeutic agent within the composition.

The "at least a second biological and/or therapeutic agent" will often be a therapeutic agent, but it need not be. For example, the at least a second biological agent may be a pharmaceutically acceptable excipient, diluent or vehicle; an anti-fungal or anti-bacterial agent; or other biological agent associated with the preparation and/or storage of pharmaceuticals. The second biological agent may also be a diagnostic agent, which is maintained separately from the alkylammonium thiomolybdate therapeutic agent.

Where the at least a second biological agent is a therapeutic agent, such an agent will typically be selected on the basis of the condition to be treated. For example, anti-arthritic compounds may be employed in arthritis treatment, and such like. Such "combined" compositions and pharmaceutical compositions of the invention include mixtures or "cocktails", as well as distinct formulations that are packaged for sale and/or use within the invention.

In the combined compositions, pharmaceuticals, cocktails, kits, and the associated methods, medicaments and first and second medical uses of the invention, the combinations are "therapeutically effective combinations". Thus, the intended practice of the invention involves the prior, simultaneous or subsequent administration of the second therapeutic agent so that a combined therapeutically effective amount of agents results *in vivo*, irrespective of the time of administration, and aside from whether the combined therapeutically effective amount is an incremental, additive or synergistic amount.

Certain preferred second therapeutic agents are second anti-angiogenic agents, or "second, distinct anti-angiogenic agents", with the at least a first alkylammonium thiomolybdate compound being the first anti-angiogenic agent within the composition. Exemplary second anti-angiogenic agents include other thiomolybdate compounds not associated with alkylammonium groups, such as ammonium tetrathiomolybdate itself. Additional thiomolybdate compounds associated with at least a first carbohydrate molecule, such as a monosaccharide, disaccharide, trisaccharide, oligosaccharide or polysaccharide, are

further examples of the agents that may be used in combination with the alkylammonium thiomolybdate compounds of the invention.

Further exemplary anti-angiogenic agents for combined use in the compositions and pharmaceutical compositions of the invention are those selected from Table 2. Anti-VEGF antibodies are another group of suitable anti-angiogenic agents. By way of example, the at least a second anti-angiogenic agent may be an anti-angiogenic agent selected from the group consisting of angiostatin, endostatin, trientine and pencillamine. Other effective second anti-angiogenic agents for combined use in the invention are zinc compounds.

Where the condition associated with aberrant angiogenesis to be treated is cancer, the at least a second therapeutic agent may be at least a second anti-cancer agent, *i.e.*, "at least a second, distinct anti-cancer agent". In such embodiments, the at least a first alkylammonium thiomolybdate compound will again be the "at least a first anti-cancer agent".

Exemplary second anti-cancer agents include those selected from chemotherapeutic agents, radiotherapeutic agents, immunotoxins, apoptosis-inducing agents, distinct anti-angiogenic agents and distinct agents that bind copper. Second anti-cancer agents again include zinc compounds.

As used herein, the term "chemotherapeutic agent" is used to refer to a classical chemotherapeutic agent or drug used in the treatment of malignancies. This term is used for simplicity notwithstanding the fact that other compounds, including immunotoxins, may be technically described as a chemotherapeutic agent in that they exert an anti-cancer effect. However, "chemotherapeutic" has come to have a distinct meaning in the art and is being used according to this standard meaning. "Chemotherapeutics" in the context of the present application therefore do not generally refer to immunotoxins, radiotherapeutic agents and such like, despite their operational overlap.

A number of exemplary chemotherapeutic agents are known in the art and further disclosed herein. Those of ordinary skill in the art will readily understand the uses and appropriate doses of chemotherapeutic agents, although the doses may well be reduced when used in combination with the present invention. New drugs that may also be termed

"chemotherapeutic agents" are agents that induce apoptosis. Any one or more of such drugs, including genes, vectors and antisense constructs, as appropriate, may also be used in conjunction with the present invention.

The invention also provides a range of therapeutic kits. Certain therapeutic kits comprise, in one example, at least a first pharmaceutical composition comprising a pharmaceutically acceptable excipient and a biologically or therapeutically effective amount of at least a first alkylammonium thiomolybdate compound. Other kits comprise, in another example, a biologically or therapeutically effective amount of at least a first alkylammonium thiomolybdate compound and instructions for administering the compound in the treatment or prevention of a disease associated with aberrant vascularization, such as instructions for administering the compound in the treatment or prevention of cancer. Preferably, either of these kits will comprise at least a first container for the pharmaceutical composition or composition.

The kits may also comprise a biologically or therapeutically effective amount of at least one diagnostic component or at least a second biological or therapeutic agent, such as an anti-angiogenic agent or anti-cancer agent. Any one or more of the second biological or therapeutic agents described above may be used in such kits.

In such kits, any combined therapeutic agents may be comprised within a single container or container means, or comprised within distinct containers or container means. Cocktails will generally be admixed together for combined use. Diagnostic and assay components will be included within distinct containers or container means.

In particular embodiments, the kits of the invention will comprise at least one component of an assay system for determining serum ceruloplasmin levels. Preferably, the kits will comprise each component of an assay system necessary for determining serum ceruloplasmin levels. Such components, or the entire assay system, will be comprised in a separate container from the at least a first tetraalkylammonium tetrathiomolybdate compound of the kit. In preferred embodiments, such kits may further comprise additional instructions, such as those for adjusting alkylammonium thiomolybdate therapy based upon the serum ceruloplasmin levels determined for a given patient.

Yet further kits of the invention include those that comprise a biologically or diagnostically effective amount of at least one component of an assay system for detecting or diagnosing cancer, *i.e.*, "a cancer diagnostic component". Entire cancer diagnostic systems or assays will be preferred. Such components or systems will be comprised within a composition distinct from the at least a first tetraalkylammonium tetrathiomolybdate compound.

The compounds, compositions, pharmaceutical formulations, kits and combinations of the invention are particularly suited for use in binding copper and forming agent-copper-protein complexes, and can thus be effectively used in treating or preventing a range of diseases and disorders associated with or characterized by aberrant vascularization or angiogenesis.

Accordingly, the invention also provides for the use of one or more alkylammonium thiomolybdate compounds in the manufacture of a medicament for treating or preventing a disease characterized by aberrant vascularization or angiogenesis. A particular aspect of the invention is the use of at least a first alkylammonium thiomolybdate compounds in the manufacture of a medicament for treating or preventing a disease characterized by aberrant vascularization or angiogenesis in a human subject. Another particular aspect of the invention is the use of an alkylammonium thiomolybdate compound that binds copper and forms a thiomolybdate compound-copper-protein complex in the manufacture of a medicament for treating or preventing a disease characterized by aberrant vascularization or angiogenesis, optionally in a human subject.

These aspects of the invention give rise to further inventive kits. For example, therapeutic kits, preferably for administration to a human subject, which kits comprise:

(a) a medicament manufactured according to the present invention, *i.e.*, a medicament comprising at least a first alkylammonium thiomolybdate compound; and

(b) means for determining serum ceruloplasmin (Cp) levels, preferably for determining serum ceruloplasmin levels in a human subject.

Additional kits of the invention are those that comprise:

(a) a loading dose of a medicament manufactured according to the present invention, *i.e.*, a medicament comprising at least a first alkylammonium thiomolybdate compound, wherein the loading dose reduces the serum ceruloplasmin level to below about 20% of the level prior to administration of the medicament, and

(b) a maintenance dose of a medicament manufactured according to the present invention, *i.e.*, a medicament comprising at least a first alkylammonium thiomolybdate compound, wherein the maintenance dose maintains the serum ceruloplasmin level at below about 20% of the level prior to administration of the medicament.

In terms of methods, the invention further provides methods of treating or preventing diseases associated with aberrant vascularization or angiogenesis. As used herein, the terms "aberrant vascularization" and "aberrant angiogenesis" will be understood to mean abnormal neovascularization, including the formation of new blood vessels, larger blood vessels, more branched blood vessels (intussusception), and any and all mechanisms that lead to inappropriate or increased blood carrying capacity to a diseased tissue or site. The agents of the present invention will be understood to counteract "aberrant vascularization" or "aberrant angiogenesis", irrespective of the actual mechanism of action.

The "anti-angiogenic therapy" provided by the invention may be applied in animals and patients that have, or are at risk for developing, any disease or disorder characterized by undesired, inappropriate, aberrant, excessive and/or pathological vascularization or angiogenesis. It is well known to those of ordinary skill in the art that as the processes underlying angiogenesis are essentially the same irrespective of the surrounding tissue, and as aberrant angiogenesis occurs in a wide range of diseases and disorders, a given anti-

angiogenic therapy, once shown to be effective in any acceptable model system, can be used to treat the entire range of diseases and disorders connected with angiogenesis.

The methods, medicaments and medical uses of the invention are particularly intended for use in animals and patients that have, or are at risk for developing, any form of vascularized tumor; macular degeneration, including age-related macular degeneration; arthritis, including rheumatoid arthritis; atherosclerosis and atherosclerotic plaques; diabetic retinopathy and other retinopathies; thyroid hyperplasias, including Grave's disease; hemangioma; neovascular glaucoma; and psoriasis.

These methods, medicaments and medical uses may further be used in the treatment of animals and patients that have, or are at risk for developing, arteriovenous malformations (AVM), meningioma, and vascular restenosis, including restenosis following angioplasty. Other intended targets of the therapeutic methods and uses are animals and patients that have, or are at risk for developing, angiofibroma, dermatitis, endometriosis, hemophilic joints, hypertrophic scars, inflammatory diseases and disorders, pyogenic granuloma, scleroderma, synovitis, trachoma and vascular adhesions.

As disclosed in U.S. Patent No. 5,712,291, specifically incorporated herein by reference, each of the foregoing somewhat preferred treatment groups are by no means exhaustive of the types of conditions that are to be treated by the present invention. U.S. Patent No. 5,712,291 is incorporated herein by reference for purposes including identifying a number of other conditions that may be effectively treated by anti-angiogenic therapies; to show that the treatment of all angiogenic diseases represents a unified concept, once a defined category of anti-angiogenic compounds have been disclosed (in this case, alkylammonium thiomolybdate compounds); and to show that the treatment of all angiogenic diseases is enabled by data from only a single model system.

In yet further aspects, and as disclosed in U.S. Patent No. 5,712,291, incorporated herein by reference, the methods, medicaments and medical uses of the present invention are intended for the treatment of animals and patients that have, or are at risk for developing, abnormal proliferation of fibrovascular tissue, acne rosacea, acquired immune deficiency syndrome, artery occlusion, atopic keratitis, bacterial ulcers, Bechets disease, blood borne

tumors, carotid obstructive disease, chemical burns, choroidal neovascularization, chronic inflammation, chronic retinal detachment, chronic uveitis, chronic vitritis, contact lens overwear, corneal graft rejection, corneal neovascularization, corneal graft neovascularization, Crohn's disease, Eales disease, epidemic keratoconjunctivitis, fungal ulcers, Herpes simplex infections, Herpes zoster infections, hyperviscosity syndromes, Kaposi's sarcoma, leukemia, lipid degeneration, Lyme's disease, marginal keratolysis, Mooren ulcer, Mycobacteria infections other than leprosy, myopia, ocular neovascular disease, optic pits, Osler-Weber syndrome (Osler-Weber-Rendu, osteoarthritis, Pagets disease, pars planitis, pemphigoid, phylectenulosis, polyarteritis, post-laser complications, protozoan infections, pseudoxanthoma elasticum, pterygium keratitis sicca, radial keratotomy, retinal neovascularization, retinopathy of prematurity, retrolental fibroplasias, sarcoid, scleritis, sickle cell anemia, Sogrens syndrome, solid tumors, Stargarts disease, Steven's Johnson disease, superior limbic keratitis, syphilis, systemic lupus, Terrien's marginal degeneration, toxoplasmosis, trauma, tumors of Ewing sarcoma, tumors of neuroblastoma, tumors of osteosarcoma, tumors of retinoblastoma, tumors of rhabdomyosarcoma, ulceritive colitis, vein occlusion, Vitamin A deficiency and Wegeners sarcoidosis.

The present invention further provides methods, medicaments and medical uses for the treatment of animals and patients that have, or are at risk for developing, arthritis, in common with the treatment of arthritis described in U.S. Patent No. 5,753,230, specifically incorporated herein by reference. U.S. Patent No. 5,972,922 is also specifically incorporated herein by reference to even further exemplify the application of anti-angiogenic strategies to the treatment of undesired angiogenesis associated with diabetes, parasitic diseases, abnormal wound healing, hypertrophy following surgery, burns, injury or trauma, inhibition of hair growth, inhibition of ovulation and corpus luteum formation, inhibition of implantation and inhibition of embryo development in the uterus. U.S. Patent No. 5,639,757 is further specifically incorporated herein by reference to exemplify the use of anti-angiogenic strategies to the general treatment of graft rejection. All of the foregoing conditions are therefore contemplated for treatment by the methods and uses of the present invention.

Although the treatment of each of the foregoing diseases is enabled within the present, unified invention, a particularly preferred aspect of the methods, medicaments and medical

uses of the invention is application of anti-angiogenic therapy to animals and patients that have, or are at risk for developing, a vascularized solid tumor, a metastatic tumor or metastases from a primary tumor.

In the context of the present invention, the term "a vascularized tumor" most preferably means a vascularized, malignant tumor, solid tumor or "cancer". In terms of cancer treatment, the compositions and methods of the invention can be used to treat all forms of solid tumors having a vascular component. Solid tumors, such as carcinomas and sarcomas, are exemplary of the types of tumors particularly amenable to treatment with the instant compositions and methods. Exemplary types of tumors that may be prevented or treated using the present invention include, but are not limited to, renal, lung, breast, colon, prostate, stomach, liver, pancreas, esophagus, brain and larynx tumors, as well as angiosarcomas and chondrosarcomas.

Tumors of various sizes may also be treated using this invention. Thus, small tumors, including metastatic tumors, exemplified by tumors that are about 1 cm in diameter or less, medium or moderate tumors, exemplified by tumors that are between about 1 cm and about 5 cm in diameter, and large tumors, exemplified by tumors that are about 5 cm in diameter or greater are contemplated for treatment using the present invention. The tumors may be primary or metastatic tumors, or both.

As the compositions and methods of the present invention are applicable to treating various tumors irrespective of the phenotype of the tumor cells, patients having more than one type of tumor will be effectively treated by the invention. Thus, patients having at least a first and second, third or further, distinct type of tumor are contemplated for treatment using the present invention, as exemplified by those having a breast tumor and a chondrosarcoma or a renal tumor and a lung tumor.

Exemplary methods of the invention comprise administering to an animal or patient having or at risk for developing a disease associated with aberrant vascularization, including macular degeneration, rheumatoid arthritis and cancer, a pharmaceutical composition comprising a biologically or therapeutically effective amount of at least a first alkylammonium thiomolybdate compound. Any one or more of the foregoing

alkylammonium thiomolybdate compounds may be used in such methods, including tetraalkylammonium tetrathiomolybdate compounds, such as tetramethylammonium tetrathiomolybdate, tetraethylammonium tetrathiomolybdate or tetrabutylammonium tetrathiomolybdate, with tetrapropylammonium tetrathiomolybdate being preferred in certain embodiments.

In certain embodiments, the invention provides methods of treating or preventing diseases associated with aberrant vascularization or angiogenesis, including macular degeneration, rheumatoid arthritis and cancer, comprising administering to an animal or patient having or at risk for developing such a disease, at least a first alkylammonium thiomolybdate compound that is substantially soluble in water and substantially stable upon exposure to air and moisture in an amount effective to release a therapeutic dose of a bioactive thiomolybdate compound upon administration to the animal or patient.

In other embodiments, the invention provides methods of treating or preventing diseases associated with aberrant vascularization or angiogenesis, including macular degeneration, rheumatoid arthritis and cancer, comprising administering to an animal or patient having or at risk for developing such a disease, at least a first alkylammonium tetrathiomolybdate compound that comprises a number of alkyl groups sufficient to substantially protect the tetrathiomolybdate from oxidation during storage prior to administration; wherein the alkylammonium tetrathiomolybdate compound releases a therapeutically effective amount of bioactive tetrathiomolybdate upon administration to the animal or patient.

In yet further embodiments, the methods of the invention include those for treating or preventing diseases associated with aberrant vascularization or angiogenesis, including macular degeneration, rheumatoid arthritis and cancer, comprising administering to an animal or patient having or at risk for developing such a disease, a pharmaceutical composition comprising at least a first tetraalkylammonium tetrathiomolybdate compound in which the alkyl groups confer substantially increased shelf-life to the tetrathiomolybdate compound; wherein the tetraalkylammonium tetrathiomolybdate compound retains solubility and releases an effective amount of substantially therapeutically active tetrathiomolybdate and substantially non-toxic alkylammonium groups upon administration to the animal or patient.

The pharmaceutical compositions of the invention may be administered to the animals or patients via any appropriate route, such as parenterally, orally, ophthalmically or such like. In certain aspects, oral administration is preferred. However, other routes of administration are contemplated, including, but not limited to, intravenous, intramuscular and subcutaneous injections; rectal, nasal, topical and vaginal administration; slow release formulations and the like.

Irrespective of the disease, the invention provides methods of delaying the onset of, or preventing, diseases associated with aberrant vascularization or angiogenesis, including macular degeneration, rheumatoid arthritis and cancer. Such methods comprise administering to an animal or patient at risk for developing a disease associated with aberrant vascularization or angiogenesis, such as macular degeneration, rheumatoid arthritis or cancer, at least a first pharmaceutical composition comprising a prophylactically effective amount of at least a first alkylammonium thiomolybdate compound. In such preventative or prophylactic methods, a patient at risk for developing a disease associated with aberrant vascularization or angiogenesis, such as macular degeneration, rheumatoid arthritis or cancer, may be "selected" or identified, such as by identifying relatives of existing patients, identifying susceptible subjects by genetic testing and such like.

The "prophylactically effective amounts" are amounts of the one or more alkylammonium thiomolybdate compounds effective to delay the onset of, or substantially prevent, diseases associated with aberrant vascularization or angiogenesis, such as macular degeneration, rheumatoid arthritis and cancer, upon administration to an animal or patient.

The present invention also provides overt therapeutic methods, which are applicable to the entire range of angiogenic diseases. The invention thus provides methods of treating a disease associated with aberrant vascularization or angiogenesis, such as macular degeneration, rheumatoid arthritis and cancer, comprising administering to an animal or patient having a disease associated with aberrant vascularization or angiogenesis, such as macular degeneration, rheumatoid arthritis or cancer, at least a first pharmaceutical composition comprising a therapeutically effective amount of at least a first alkylammonium thiomolybdate compound.

"Therapeutically effective amounts" of the invention are thus amounts of the one or more alkylammonium thiomolybdate compounds effective to delay or substantially prevent the progression of, and/or to alleviate the symptoms of, a disease associated with aberrant vascularization or angiogenesis, such as macular degeneration, rheumatoid arthritis or cancer, upon administration to an animal or patient. For example, therapeutically effective amounts may slow the growth of diseased tissue, *e.g.*, a tumor; substantially arrest such growth; specifically induce necrosis in at least a portion of the diseased tissue or tumor; and/or induce disease regression or remission upon administration to an animal or patient.

Such therapeutic effects are achieved while preferably exhibiting negligible or manageable adverse side effects on normal, healthy tissues of the animal or patient. Thus, the "therapeutically effective amount" can vary from animal to animal or patient to patient, depending on a number of factors including, but not limited to, the extent of disease and the size of patient. All such dosing issues can be routinely addressed by the attending physician in light of the present disclosure.

In certain aspects of the invention, the biologically or therapeutically effective amount of the at least a first alkylammonium thiomolybdate compounds is between about 10 mg and about 300 mg per patient. In other aspects, the biologically or therapeutically effective amounts are between about 20 mg and about 200 mg per patient over a therapeutically effective time or period. In general, the agent is administered to the patient daily, and thus in these embodiments of the invention, the biologically or therapeutically effective amount of the at least a first agent is between about 10 mg and about 300 mg, or between about 20 mg and about 200 mg, per patient per day. Certain preferred doses are between about 125 mg and about 190 mg, and between about 150 mg and about 180 mg.

"Between about 10 mg and about 300 mg" includes all values in this range, and thus includes amounts of about 20 mg, about 25 mg, about 30 mg, about 40 mg, about 50 mg, about 60 mg, about 70 mg, about 75 mg, about 80 mg, about 90 mg, about 100 mg, about 110 mg, about 120 mg, about 125 mg, about 130 mg, about 140 mg, about 150 mg, about 160 mg, about 170 mg, about 180 mg, about 190 mg, about 200 mg, about 210 mg, about 220 mg, about 230 mg, about 240 mg, about 250 mg, about 260 mg, about 270 mg, about

280 mg, about 290 mg and about 295 mg. "About" will be understood to include values above and below the given number. Thus, "about 20 mg" will be understood to include about 18 mg, about 19 mg, about 21 mg and about 22 mg or so, and "about 200 mg" will be understood to include about 201 mg, about 202 mg, about 203 mg and about 204 mg or so.

In other embodiments of the invention, the biologically or therapeutically effective amount of the at least a first agent is between about 20 mg and about 190 mg, between about 20 mg and about 180 mg, between about 20 mg and about 170 mg, between about 20 mg and about 160 mg, between about 20 mg and about 150 mg, between about 20 mg and about 140 mg, between about 20 mg and about 130 mg, between about 20 mg and about 120 mg, between about 20 mg and about 110 mg, between about 20 mg and about 100 mg, between about 20 mg and about 90 mg, between about 20 mg and about 80 mg, between about 20 mg and about 70 mg, between about 20 mg and about 60 mg, between about 20 mg and about 50 mg, between about 20 mg and about 40 mg, between about 20 mg and about 30 mg, between about 30 mg and about 200 mg, between about 40 mg and about 200 mg, between about 50 mg and about 200 mg, between about 60 mg and about 200 mg, between about 70 mg and about 200 mg, between about 80 mg and about 200 mg, between about 90 mg and about 200 mg, between about 100 mg and about 200 mg, between about 110 mg and about 200 mg, between about 120 mg and about 200 mg, between about 130 mg and about 200 mg, between about 140 mg and about 200 mg, between about 150 mg and about 200 mg, between about 160 mg and about 200 mg, between about 170 mg and about 200 mg, between about 180 mg and about 200 mg, between about 190 mg and about 200 mg, between about 30 mg and about 190 mg, between about 40 mg and about 180 mg, between about 50 mg and about 170 mg, between about 60 mg and about 160 mg, between about 70 mg and about 150 mg, between about 80 mg and about 140 mg, between about 90 mg and about 130 mg, between about 100 mg and about 120 mg, between about 125 mg and about 200 mg or between about 150 mg and about 180 mg per patient per day.

The foregoing values can also be expressed in terms of mg/kg of body weight. As described above, the biologically or therapeutically effective amount can vary depending on the size of the animal or human patient. However, taking the average weight of a human male as about 70 kg, the biologically or therapeutically effective amount of the compounds can be readily calculated in mg/kg.

The biologically and therapeutically effective amounts can also be effectively expressed in terms of copper reduction levels. As such, the invention preferably concerns the administration of at least a first alkylammonium thiomolybdate compound to an animal or patient with a disease associated with aberrant vascularization or angiogenesis, including macular degeneration, rheumatoid arthritis and cancer, in an amount and for a period of time effective to reduce the level of copper in the animal or patient to between about 10% and about 40% of the level of copper in the animal or patient prior to administration of the alkylammonium thiomolybdate compound. Copper reduction to levels of between about 15% and about 30%, between about 10% and about 20%, or between about 15% and about 20% are particularly effective, with reduction to about 20% of the level prior to treatment being effective for most animals and patients.

The levels of copper in animals and patients may be readily determined, and a preferred method is to determine the level of serum ceruloplasmin.

In further embodiments, the initial treatment of the invention is followed by subsequently administering to the animal or patient an amount of a copper binding agent effective to substantially maintain the reduced copper levels in the animal or patient. Such subsequent administration is preferably given in amounts and for periods of time effective to maintain the level of copper in the animal or patient at about 10%-20% of the level prior to initial treatment.

Thus, the invention includes treatment methods wherein a loading dose of at least a first alkylammonium thiomolybdate compound is first administered to the animal or patient in an amount and for a period of time effective to initially reduce the level of copper in the animal or patient to about 20%-40% of the level prior to administration; and wherein a maintenance dose of a copper binding agent is subsequently administered to the animal or patient in an amount and for a period of time effective to maintain the level of copper in the animal or patient at about 10%-20% of the level prior to initial treatment.

These methods of the invention are applicable to treating or preventing diseases associated with aberrant vascularization or angiogenesis, including macular degeneration,

rheumatoid arthritis and cancer. Such methods comprise administering to an animal or patient having or at risk for developing such a disease:

(a) an amount of at least a first alkylammonium thiomolybdate compound sufficient to effectively reduce the level of copper in the animal or patient, preferably to between about 10% and about 40% of the pre-treatment copper levels, more preferably to about 20% of the pre-treatment copper levels; and

(b) substantially maintaining the reduced copper levels in the animal or patient.

In all such embodiments, the subsequently administered copper binding agent may be any agent effective to bind and reduce copper levels in the animal. Suitable copper binding agents are thiomolybdate compounds, including the alkylammonium thiomolybdate compounds of the primary treatment. However, other copper binding agents may be effectively used, particularly zinc compounds, such as zinc acetate. Copper levels are preferably indicated by serum ceruloplasmin levels.

Any of the foregoing methods may further comprise administering to the animal or patient a therapeutically effective amount of at least a second therapeutic agent. The second therapeutic agent may be a second anti-angiogenic agent or a second anti-cancer agent. Second therapeutic agents may also be agents for combating the disease to be treated by means other than anti-angiogenesis, such as anti-arthritic compounds. Exemplary second anti-angiogenic agents are distinct copper chelating agents and zinc compounds. Exemplary second anti-cancer agents are chemotherapeutic agents, radiotherapeutic agents, distinct anti-angiogenic agents and apoptosis-inducing agents.

The at least a second therapeutic, anti-angiogenic or anti-cancer agent may be administered to the animal or patient substantially simultaneously with the at least a first alkylammonium thiomolybdate compound, such as from a single pharmaceutical composition or from two pharmaceutical compositions administered closely together.

Alternatively, the at least a second therapeutic, anti-angiogenic or anti-cancer agent may be administered to the animal or patient at a time sequential to the administration of the at least a first alkylammonium thiomolybdate compound. "At a time sequential", as used herein, means "staggered", such that the at least a second agent is administered to the animal or patient at a time distinct to the administration of the alkylammonium thiomolybdate compound.

Generally, the two agents are administered at times effectively spaced apart to allow the two agents to exert their respective therapeutic effects, *i.e.*, they are administered at "biologically effective time intervals". The at least a second agent may be administered to the animal or patient at a biologically effective time prior to the alkylammonium thiomolybdate compound, or at a biologically effective time subsequent to that compound. Other combined treatments, including surgical resection and radiotherapy, *e.g.*, X-ray therapy, are also included in the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. The crystal packing projection of $(n\text{-}Pr_4N)_2MoS_4$ (TP-TM).

FIG. 2. The X-ray powder pattern calculated on the basis of atomic coordinates obtained from the single-crystal X-ray structure determination.

FIG. 3. The X-ray powder pattern obtained experimentally from the crystalline $(n\text{-}Pr_4N)_2MoS_4$ (TP-TM).

FIG. 4. Enhanced stability of TP-TM versus TM (AmmTM). Stability of each compound was studied under conditions that exacerbate instability, *i.e.* the drugs were in

open Petri dishes at room temperature. The percentage activity is plotted as a function of time in days.

FIG. 5. TP-TM inhibits *in vivo* growth of human tumor xenografts as effectively as TM. 10^6 breast cancer cells were injected in the mammary fat pad of 4 groups of athymic, nude mice (5 mice per group). Control mice received no treatment. The 3 experimental groups began treatment at the indicated time, and received either 1 mg/day of the original TM, AmmTM; 1 mg/day of TP-TM, tetrapropyl-tetrathiomolybdate (indicated as "TP 1 mg" on the figure); or 1.5 mg/day of TP-TM ("TP 1.5 mg" on the figure). The error bars were within 5% of represented values. The growth curves of the treated animals are significantly different from the control animals, $p < 0.01$. The TP-TM (TP) growth curves are statistically indistinguishable from the TM curves.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Solid tumors account for more than 90% of all cancers in man (Shockley *et al.*, 1991). The therapeutic uses of monoclonal antibodies and immunotoxins have been investigated in the therapy of lymphomas and leukemias (Lowder *et al.*, 1987; Vitetta *et al.*, 1991), but have been disappointingly ineffective in clinical trials against solid tumors (Byers and Baldwin, 1988; Abrams and Oldham, 1985).

A principal reason for the ineffectiveness of antibody-based treatments is that macromolecules are not readily transported into solid tumors (Sands, 1988; Epenetos *et al.*, 1986). Even when these molecules get into the tumor mass, they fail to distribute evenly due to the presence of tight junctions between tumor cells (Dvorak *et al.*, 1991), fibrous stroma (Baxter *et al.*, 1991), interstitial pressure gradients (Jain, 1990) and binding site barriers (Juweid *et al.*, 1992).

All too often in cancer control, a high-functioning individual is coping with the high likelihood of a clinical cancer diagnosis or the inexorable progression of largely asymptomatic cancer to a lethal stage. At the cellular level, in spite of the diversity of the clinical settings, the incipient and existing tumors will all require new blood vessel growth or angiogenesis to affect the quality of life of their hosts. Therefore, the development of

successful anti-angiogenic therapies may have the effect of preventing an emerging tumor from becoming clinically relevant, or may allow a prolonged asymptomatic state with stable metastatic disease. In addition, such therapy may also have the effect of down-staging overt tumors.

It has been known since the 1970's that copper is a co-factor in angiogenesis. Many key angiogenic mediators such as FGF, angiogenin, and SPARC bind or interact with copper in their pro-angiogenic state. The concept of anti-angiogenic treatment for solid tumors (Folkman, 1972; 1995c; 1997), has a firm rationale and shows efficacy in animal tumor models (Volpert *et al.,* 1996; Millauer *et al.,* 1996; Warren *et al.,* 1995; Borgstom *et al.,* 1996; 1998; Yuan *et al.,* 1996; O'Reilly *et al.,* 1997; Benjamin *et al.,* 1999 and Merajver *et al.,* 1998). Compounds that interfere with critical steps in the angiogenesis cascade are reaching the clinic (Marshall *et al.,* 1997). The steps required for successful tumor angiogenesis at the primary and metastatic sites are diverse, and they depend on an imbalance between angiogenesis activators (Iruela-Arispe and Dvorak, 1997; Hanahan and Folkman, 1996) such as VEGF and bFGF, and inhibitors such as thrombospondin-1 (TSP-1) (Volpert *et al.,* 1995; Salnikow *et al.,* 1997; Guo *et al.,* 1997; Schapira and Schapira, 1983; Qian *et al.,* 1997), angiostatin, (O'Reilly *et al.,* 1994; Lannutti *et al.,* 1997; Sim *et al.,* 1997) and endostatin (O'Reilly *et al.,* 1997). The relative importance of the different angiogenesis modulating molecules in different tissues may determine the relative potency of anti-angiogenic compounds to elicit a response both at the primary and metastatic sites.

It has been amply demonstrated that copper is required for angiogenesis (Parke *et al.,* 1988; Raju *et al.,* 1982; Ziche *et al.,* 1982). Brem *et al.* (1990a, b), and more recently Yoshida *et al.* (1995) attempted to use an anti-copper approach in the treatment of tumors in animal studies. They have used a copper deficient diet combined with penicillamine therapy, called CDPT. The studies showed relative inhibition of growth of rabbit VX2 carcinoma in the rabbit brain (Brem *et al.*, 1990b) and rat 9L gliosarcoma in the rat brain (Brem *et al.*, 1990a, Yoshida *et al.*, 1995) as a result of such treatment, but, significantly, reported no improvement in overall survival. The animals in Yoshida *et al.* were sacrificed before overall survival could be assessed. In the Brem *et al.* studies, death was due to accompanying intracerebral edema, which was severe enough in treated animals to cause death at the same rate as the untreated tumor did in control animals. Furthermore, in contrast to the rabbit brain

model, CDPT failed to inhibit tumor growth and vascularization of the VX2 carcinoma in the thigh muscle and in metastases to lung. The lack of improvement in survival in brain tumor implant models and the lack of effect in non-brain tumors seems to have discouraged further work in this area.

The inventors reasoned that it would be very desirable to develop an anti-angiogenic strategy that would affect multiple activators of angiogenesis, in order for it to be generally applicable to human tumors. Many anti-angiogenic proposals are directed against a single target. Since copper is a required co-factor for the function of many key mediators of angiogenesis, such as bFGF (Watanabe *et al.,* 1990; Engleka and Maciag, 1994; Shing, 1988; Patstone and Maher, 1996), VEGF, and angiogenin (Badet *et al.,* 1989), the inventors developed an anti-angiogenic strategy for the treatment of cancer and other diseases characterized by aberrant angiogenesis based on the modulation of total-body copper status. The underlying basis of the inventors' early work was to identify a window of copper deficiency within swhich angiogenesis is impaired, but other copper dependent cellular processes are not affected enough to cause clinical toxicity. The inventors succeeded in their clinical objectives, despite the documented failures of others using animal models.

The development of safe and effective prophylactic or therapeutic agents that reduce the level of copper *in vivo* has been an ongoing problem in the art. The inventors' hypothesis of an anti-copper, anti-angiogenic approach to cancer therapy is that the level of copper required for angiogenesis is higher relative to that required for essential copper dependent cellular functions, such as heme synthesis, cytochrome function, and incorporation of copper into enzymes and other proteins. The inventors first reasoned that the unique and favorable characteristics of tetrathiomolybdate (TM) as an anti-copper agent, compared to other anti-copper drugs, could make TM a non-toxic, efficacious new weapon in an anti-copper, anti-angiogenic therapy.

As described in detail below, for the past 20 years the inventors have developed new anti-copper therapies for Wilson's disease, an autosomal recessive disease of copper transport that results in abnormal copper accumulation and toxicity. One of the drugs currently being used is TM, which shows unique and desirable properties of fast action, copper-specificity, and low toxicity (Brewer *et al.,* 1991a; 1994b; 1996), as well as a unique mechanism of

action. TM forms a stable tripartite complex with copper and protein. If given with food, it complexes food copper with food protein and prevents absorption of copper from the gastrointestinal tract. There is endogenous secretion of copper in saliva and gastric secretions associated with food intake, and this copper is also complexed by TM when it is taken with meals, thereby preventing copper re-absorption. Thus, patients are placed in a negative copper balance immediately when TM is given with food. If TM is given between meals, it is absorbed into the blood stream, where it complexes either free or loosely bound copper with serum albumin. This TM-bound copper fraction is no longer available for cellular uptake and has no known biological activity.

The early studies of the present inventors' indeed showed TM to be a safe and effective anti-cancer agent. TM showed efficacy in impairing the development of *de novo* mammary tumors in Her2-neu transgenic mice (Example 2), and showed no clinically overt toxicity as copper levels were decreased to 10% of baseline. The present disclosure also includes details of the first human trial of an anti-copper approach to anti-angiogenesis therapy based on the use of tetrathiomolybdate in patients with metastatic cancer (Examples 3 and 4). The phase I trial of TM, yielding information on dose, dose response, evaluation of copper status in patients, toxicity and efficacy, is surprisingly beneficial, particularly in light of the disappointing animal studies conducted by others (Brem *et al.*, 1990a, b; Yoshida *et al.*, 1995).

Although the inventors' conceptual breakthrough, and the seminal work that developed the concept into clinical success cannot be understated, the straightforward and economical development of widespread treatment methods would be significantly advanced by the development of improved, "second generation" compounds, particularly those with enhanced stability and shelf life. The present invention provides a range of such compounds, which have significantly improved stability upon exposure to air and moisture, but which retain solubility and provide the same therapeutic responses upon administration *in vivo*.

I. Wilson's Disease

A. Background

Wilson's disease is an autosomal recessive disorder of copper metabolism. In this disorder, the excretion of copper into the bile appears to be defective, and there is reduced

hepatic incorporation of copper into ceruloplasmin, leading to an accumulation of copper in plasma and most body tissues. Wilson's disease usually leads to hepatic and/or neurologic dysfunction.

The therapy of Wilson's disease can be divided into two broad categories (Brewer and Yuzbasiyan-Gurkan, 1992a). These are initial therapy in acutely ill patients, and maintenance therapy. Initial therapy is that period of time during which a newly presenting patient is still suffering from acute copper toxicity, generally the first few weeks to months of therapy. Maintenance therapy is essentially the rest of the patient's life, or that period of time after the copper levels have been brought down to a subtoxic threshold, and the patient is on therapy simply to prevent the recurrence of copper accumulation and copper toxicity.

For the maintenance therapy of Wilson's disease three drugs are currently available. These include the oldest available drug, penicillamine (Walshe, 1956), a drug called trien or trientine which was developed for patients who are intolerant of penicillamine (Walshe, 1982), and zinc acetate (Brewer and Yuzbasiyan-Gurkan, 1992a, b; Brewer *et al.*, 1983; Hill *et al.*, 1987; Hill *et al.*, 1986; Brewer *et al.*, 1987b, c, d; Yuzbasiyan-Gurkan *et al.*, 1989; Brewer *et al.*, 1989; Lee *et al.*, 1989; Brewer *et al.*, 1990; Brewer *et al.*, 1991b; Brewer and Yuzbasiyan-Gurkan, 1989; Brewer *et al.*, 1992a, b; Yuzbasiyan-Gurkan *et al.*, 1992; Brewer *et al.*, 1993a, b, c, d; Brewer, 1993; Hoogenraad *et al.*, 1978; Hoogenraad *et al.*, 1979; Hoogenraad *et al.*, 1987). Zinc provides an effective maintenance therapy with a very low level of toxicity.

About 2/3 of patients who present with Wilson's disease present with symptoms referable to the brain (Brewer *et al.*, 1992a; Scheinberg and Sternlieb, 1984; Danks, 1989). These can be neurologic symptoms or symptoms of psychiatric nature in the beginning, with neurologic symptoms later. Therapy for these patients is not nearly as straightforward as it is for maintenance phase patients. The inventors have found that approximately 50% of these patients who are treated with penicillamine become worse rather than better (Brewer *et al.*, 1987a). Half of these patients who worsen, or about 25% of the original sample, never recover to their pre-penicillamine baseline. In other words, penicillamine has induced additional irreversible damage.

The mechanisms of this worsening are not known with certainty although it is likely that the mobilization of hepatic copper by the drug further elevates brain copper. The inventors have shown that this can occur in a rat model. Regardless of the mechanism, neurologically-presenting patients very often end up much worse off after being treated initially with penicillamine. In fact, even presymptomatic patients can develop neurologic disease after being initiated on penicillamine (Glass *et al.*, 1990; Brewer *et al.*, 1994a). It is not known whether trientine exhibits the phenomenon of neurological worsening when used as initial therapy, because it has not been used very much in this kind of situation. It would not be surprising if it exhibited this problem to some degree because of it's similar mechanism of action to that of penicillamine, but it might be much less, because its effects on copper seem to be somewhat gentler.

Zinc is not an ideal agent for the initial treatment for this type of patient. Zinc has a relatively slow onset of action, and produces only a modest negative copper balance. Thus, during the several months required for zinc to bring copper down to a subtoxic threshold, patients may be at risk for further copper toxicity and worsening of their disease.

B. Results of TM Therapy

The inventors have carried out an open label study of the use of TM for initial treatment of neurologically presenting Wilson's disease patients for the past several years. The inventors have developed both a spectrophotometric and bioassay for the activity of the drug, to evaluate stability, and assure potency of the drug being administered (Brewer *et al.*, 1991a; Brewer *et al.*, 1994b). The drug slowly loses potency when exposed to air. Oxygen molecules exchange with the sulfur molecules, rendering the drug inactive.

The results in the first patient studied can be used to illustrate several points. For the first seven days, the patient received TM only with meals (tid with meals). This produced the immediate negative copper balance one would expect from the first mechanism of action (blockade of copper absorption when given with meals). After the first seven days, TM was given between meals as well (tid with meals, and tid between meals). This led to the immediate rise in plasma copper expected from absorption of TM into the blood, and formation of a complex of copper, TM, and albumin. The copper complexed with TM and albumin is unavailable for cellular uptake, and this copper is therefore non-toxic (Gooneratne

et al., 1981b). There is a 1:1 stochiometric relationship between molybdenum and copper in this complex. Knowing the molybdenum level in the blood, and the ceruloplasmin level (ceruloplasmin also contains copper that is non-toxic), one can calculate how much of the plasma copper is not bound to one or the other. This so-called "free copper" (non-ceruloplasmin plasma copper) is the potentially toxic copper. When reduced to zero, the plasma copper-molybdenum "gap" is closed. This took 16 days in the first patient (9 days after adding the between meal doses). Since the brain (and the other organ) free copper is in equilibrium with the blood, bringing the blood free copper down to a low level begins the process of lowering the brain level of free (toxic) copper.

The inventors have now treated a total of 51 Wilson's disease patients with TM, all of whom presented with neurological or psychiatric disease, in an open label study. These patients were all diagnosed by standard criteria. These patients had a diagnostically elevated hepatic or urine copper, usually both. Some of them were treated briefly with other agents prior to this trial. Two patients had psychiatric but not neurological symptoms.

With three exceptions in the earliest part of the study, all patients received a dose of 20 mg tid with meals, or qid with three meals and a snack. Thus, the only difference between a patient receiving 120 mg and 140 mg total dose is that the former was receiving 20 mg tid, or 60 mg, with meals, and the latter was receiving 20 mg qid, or 80 mg with meals plus a snack. The rest of the total daily dose was divided up into three equal doses and given between meals.

The total daily dose was varied considerably among the patients, from a high of 410 mg to a low of 120 mg. In the end, the inventors could discern no dose-related correlation with copper variables, nor with functional variables measured either during the study or at the one and two year time point.

Zinc administration was also used in these patients. The starting time of zinc administration was varied widely and did not correlate with copper variables, outcome variables or toxicity. Early zinc therapy should theoretically help preserve liver function. In these patients, liver function returned to normal by year 1, but since these tests don't measure the extent of tissue preservation, it seems likely that zinc was somewhat beneficial.

Measuring trichloracetic acid (TCA) soluble copper of the plasma is somewhat useful in assessing the impact of TM therapy on copper metabolism in Wilson's disease. Generally, a high proportion of plasma copper in these patients is TCA soluble (it averaged 56% in patients – which is 27 μg/dl). All of the non-ceruloplasmin plasma copper is TCA soluble, and a somewhat variable portion of the ceruloplasmin copper is also TCA soluble. Because the ceruloplasmin levels are usually rather low in Wilson's disease, most of the plasma copper is TCA soluble. The copper in the TM/albumin/copper complex in the blood is TCA insoluble. Thus, as therapy proceeds, the fraction of the plasma copper which is TCA soluble becomes smaller. During the late stages of TM therapy, the TCA soluble fraction of plasma copper of the patients averaged 15 μg/dl, a significant reduction from the starting value of 27. The TCA solubility fraction cannot be used as an absolute endpoint, for example attempting to reduce it to zero, because a small and somewhat variable soluble fraction is usually present due to plasma ceruloplasmin. However, the significant mean reduction from 27 to 15 μg/dl illustrates the beneficial effect TM therapy has on the status of the potentially toxic plasma copper in these patients. Further evidence of the desirable impact of TM therapy on copper metabolism is shown by reduction of mean urine copper values during the latter part of TM therapy, compared to baseline values.

TM impacts quickly and favorably on copper metabolism, reducing the potentially toxic copper of the blood and theoretically, the rest of the body. The primary clinical objective is to gain control over copper toxicity while not allowing clinical worsening. In other words, the prime objective is to protect all neurological function that is present at the time therapy is started. This was evaluated weekly by quantitative neurological and speech exams. Methodology and the neurology rating scale system have been published (Young *et al.*, 1986). During the weeks of TM administration, during which copper metabolism is being controlled, neurological function as evaluated by quantitative neurological exam, is protected. Only two patients (4% of the sample) showed a change of more than 5 units, the criterion for significant worsening.

During the following years, while the patients are on maintenance therapy, the brain damage previously induced by copper is at least partially repaired. This is exemplified by the partial recovery in neurological scores seen at yearly time-points in follow-up. It is clear that

with the initial TM approach, long term recovery is excellent, most patients showing substantial neurological recovery. These excellent results are to be contrasted with penicillamine therapy. As pointed out earlier, about 50% of patients initially deteriorate on penicillamine, and that half of these, or 25% of the original sample, never recover to their pre-penicillamine baseline.

The results during the initial 8 weeks of TM therapy on quantitative speech exams are performed as described (Brewer *et al.* 1996). During the weeks of TM administration, during which copper metabolism is being controlled, neurological function as measured by quantitative speech exams is being controlled. No patient shows significant (more than 2.0 units) reduction in scores. During the following years, while the patients are on maintenance therapy, the brain damage previously induced by copper is partially repaired. This is exemplified by the partial recovery in speech scores over years of follow-up. Long term recovery is excellent. No patient shows significantly (more than 2.0 units) less long term function than at the time of initiation of therapy, and most show marked improvement.

Two undesirable effects from TM therapy were observed in these patients. One is a reversible anemia/bone marrow depression, which was exhibited by seven patients. The fall in hemoglobin in all of these patients was significant, averaging 3.4 g %. Three of the patients showed a reduction in platelet count and four of the patients showed a reduction in white blood cell count that may have been significant. TM was stopped in all seven cases. Except for two of the patients, stoppage was late in the 56 day course of TM.

At the time of the anemia, these patients all had zero non-ceruloplasmin plasma copper and an extremely low TCA soluble copper. The latter averaged 2.7 in these patients, and the average value for this variable in the entire group of patients was 27 at the beginning and 15 at the height of therapy. The cause of the anemia/bone marrow depression was concluded to be bone marrow depletion of copper. Since copper is required for heme synthesis and other steps in cell proliferation, it could be expected that anemia and bone marrow effect would be the first signs of copper depletion. This result from copper depletion is a well-known phenomenon.

Thus, this undesirable response to TM is not a side effect but is, rather, due to overtreatment. It is perhaps surprising that it is possible to produce even localized bone marrow copper depletion within such a short period of time in Wilson's disease, a disease in which the body is overloaded with copper. This response to TM is unique. None of the other anti-copper drugs are able to produce this effect in early therapy. Thus, this speaks to the potency of TM and the rapidity with which it can control copper levels. Its also likely that the bone marrow is especially dependent on plasma copper, and that it is the first pool that it is reduced to very low levels. At a dose of 180 mg/day or over, overtreatment occurred in 6 of 37 patients. At a dose of 150 or lower, only 1 of 13 patients exhibited overtreatment, and that was very late (53 days in the 56 day program).

The second undesirable effect of TM therapy in these patients is an elevation of transaminase values in four of the patients. The serum AST and ALT values were elevated. TM therapy was discontinued in one patient because of these elevations. During these elevations, the urine copper increases, contrary to the general trend in other patients, where it is decreasing. These data support the concept that a hepatitis is occurring, with release of copper from damaged hepatocytes. It is not clear why this hepatitis is occurring. However, untreated Wilson's disease patients have a episodic hepatitis as part of their history. Since there is little in the way of observation of untreated patients after diagnosis, no good information exists on how often episodes of transaminase elevations occur as part of the natural history of the disease.

Alternatively, the TM in some cases may be mobilizing hepatic copper at a faster rate than it can be disposed of, in which case these patients would be classified as showing a side effect of treatment. Against this is the observation in copper-poisoned sheep, in which the acute hepatitis, liver necrosis, and hemolytic anemia are rapidly corrected with high doses of TM. All four of these patients were treated with 150 mg TM/day or higher. None of the patients treated with 150 mg or lower exhibited this response. No other negative effects of TM have been observed.

II. Thiomolybdate Compounds

A. Tetrathiomolybdate

Tetrathiomolybdate (TM) is a compound made up of molybdenum atom surrounded by four sulfido groups. Discovery of the biological effects of TM began with observations on cattle and sheep, in which they developed copper deficiency when grazing on pasturages with high molybdenum (Mo) content (Ferguson *et al.*, 1943; Dick and Bull, 1945; Miller and Engel, 1960). It was established that administration of supplementary Mo impaired copper metabolism in ruminants (Macilese Ammerman *et al.*, 1969); however, Mo had little effect on non-ruminant animals such as rats (Mills *et al.*, 1958; Cox *et al.*, 1960). The answer to this puzzle came from observations which suggested that the Mo was converted to thiomolybdates in the rumen as a result of the high sulfide metabolism there, and that thiomolybdates were the active anti-copper agents (Dick *et al.*, 1975). This theory was confirmed when thiomolybdate compounds were given to rats and produced anti-copper effects (Mills *et al.*, 1981a, b; Bremner *et al.*, 1982). The tetrathio-substituted compound, TM, is the most potent of these.

The anti-copper mechanism of action of TM is two fold (Mills *et al.*, 1981a, b; Bremner *et al.*, 1982; Gooneratne *et al.*, 1981b). One mechanism operates in the GI tract, the second in the blood. In the GI tract, TM forms complexes with copper and food proteins (or other proteins), that are not absorbed. This absorption block involves not only food copper, but also the rather considerable amount of endogenously secreted copper in saliva, gastric juice and other GI tract secretions (Allen and Solomons, 1984). TM is a more effective blocker of copper absorption than zinc, since zinc acts only in those areas of the small intestine where metallothionein can be induced (Yuzbasiyan-Gurkan *et al.*, 1992). In contrast, TM works all up and down the GI tract. The other advantage of TM over zinc in this setting is that TM acts immediately. It does not have a lag period required for the induction of metallothionein.

The second effect of TM is on the blood. TM given at times away from meals is relatively well absorbed into the blood. There it forms complexes with copper and albumin, rendering the complexed copper unavailable for cellular uptake (Gooneratne *et al.*, 1981b). The normal plasma copper is in two primary pools. Most of the plasma copper in normal persons is part of the ceruloplasmin molecule. This copper is essentially unavailable for

ready exchange with cells and is considered non-toxic. The other pool of copper is more loosely bound to albumin and small molecules, such as amino acids. This pool of copper is greatly expanded during acute copper toxicity in diseases such as Wilson's disease, and is readily available for cellular uptake and is, therefore, potentially toxic (Scheinberg and Sternlieb, 1984). When TM enters the blood it complexes with this latter copper and renders it, like the ceruloplasmin copper, unavailable for cellular uptake and for further toxicity.

Very good evidence exists that TM-complexed copper is unavailable for cellular uptake. The most direct evidence is that in sheep levels of copper in the plasma which would normally be high enough to produce hemolytic anemia do not do so in the presence of TM (Gooneratne *et al.*, 1981b). It was shown that the TM bound copper does not permeate the erythrocyte. This is direct evidence that TM-complexed copper does not permeate cells.

TM and salts of TM are available; one of the preferred salts of TM is the ammonium salt. TM as purchased from Aldrich Chemical Company (catalog number W180-0; Milwaukee, WI), is a black powder that is moderately water soluble, yielding a bright red solution. TM purchased from Aldrich Chemical Company (available in one kilogram bulk lots) is certified pure for human use. The bulk drug should be stored in the absence of oxygen, or the oxygen will gradually exchange with the sulfur, rendering the drug ineffective over time. The bulk drug is therefore stored under argon. Stability assays developed by the inventors indicate that the drug is stable for several years under argon (Brewer *et al.*, 1991a). Capsules can be filled by hand, and the drug is stable in capsules for several months at room temperature.

TM acts by forming a tripartite complex with copper and protein (Mills *et al.*, 1981a, b; Bremner *et al.*, 1982). TM has two mechanisms of action. Given with meals, it complexes copper in food and endogenously secreted copper with itself and food protein, and prevents the absorption of copper. Patients can be put into an immediate negative copper balance with TM by administering it with meals. Given between meals, the TM is absorbed into the bloodstream, and complexes serum copper with itself and albumin, rapidly rendering the copper unavailable for cellular uptake. Since free copper in organs is in equilibrium with free copper of blood, free copper in the organs, and in tumor tissue, will quickly be reduced to

very low levels, if the blood copper is bound. This complex is cleared through the kidney and the liver. TM is the most potent and most rapidly acting anti-copper agent known.

B. TM Efficacy

Tetrathiomolybdate (TM) is a drug that the inventors have developed as an orphan therapy for Wilson's disease. The drug does an excellent job of gaining quick control over copper toxicity and preventing the neurological worsening that occurs 50% of the time during initial treatment with a commonly used drug for Wilson's disease, penicillamine (Brewer *et al.*, 1991a; Brewer *et al.*, 1994b; Brewer *et al.*, 1996). So far, the inventors have treated 55 Wilson's disease patients with TM, generally for an eight week period. TM thus fills a very important niche in the initial treatment of Wilson's disease. The Wilson's disease work has provided extensive experience with TM in the human, and has helped to document TM's extremely low level of toxicity in humans.

In human Wilson's disease studies, the one side effect occasionally observed is a reversible anemia, due to TM's anti-copper effects. Given in too high a dose, TM renders the bone marrow severely or totally copper deficient. Since copper is required for erythropoiesis, an anemia develops. That anemia is rapidly reversible by simply stopping TM. In the Wilson's disease studies, the overtreatment effect of TM has been diminished by simply reducing the dose to 20 mg six times per day. In humans without Wilson's disease, such as cancer patients, a level of mild copper deficiency at a pre-anemia state can be established simply by carefully monitoring ceruloplasmin (Cp) levels during TM therapy.

TM is eventually metabolized to thiomolybdates, molybdates and molybdenum oxides, so the potential toxicity of these compounds have to be considered. However, it turns out that these molybdenum compounds are quite innocuous at the levels produced from breakdown of TM used in the clinical situations described herein. About 37% of TM by weight is Mo, so in the studies described herein, up to 50 mg of Mo/day is administered for two weeks then no more than about 25 mg/day for maintenance. High doses of 350 to 1400 mg/day of Mo were previously used for 4-11 months in patients with Wilson's disease, without toxicity (Bickel *et al.*, 1957). Thus, the dose range of 25-50 mg/day poses no predictable problems, and should be entirely safe.

Considerable work on the potential toxicity of TM has been carried out in rats (Mills *et al.*, 1981a; Bremner *et al.*, 1982). At approximately 6 mg of TM per kilogram of diet rats show substantial effects on copper, including a reduction of plasma ceruloplasmin and a reduction in liver and kidney copper. At approximately 12 mg of TM all of these changes were increased and, in addition, liver Mo was increased. Mild anemia was present, and skeletal lesions were present in one of six animals. At 18 mg of TM the anemia was severe. Melanogenesis of hair was impaired, diarrhea was present, growth rate was markedly impaired, and all animals had skeletal lesions characterized by dysplasia in the epiphyseal cartilage cells of long bones, resorption of trabecular bone, and structural changes in ligaments.

It was later shown that all of the toxic effects of TM, up to 36 mg of TM per kilogram of diet, could be prevented by oral supplementation with copper, or with intraperitoneal injection of copper (Mills *et al.*, 1981b). Thus, it appears that all the toxic lesions induced by TM are due to copper deficiency induced by the TM. In support of this, almost all of the above lesions are induced by dietary copper deficiency, the two exceptions being the skeletal lesions and the enterocyte mitochondrial damage which leads to diarrhea. The reason that these last two lesions are seen with TM administration, but may not be seen in dietary copper deficiency, could be related to the severity and the rapidity of the copper deficiency induced by TM. With dietary copper deficiency there is always some contaminating copper available, and rapidly dividing cells such as the enterocyte and epiphyseal cells may obtain enough copper to prevent the lesions. The prevention of these two lesions as well as all of the other TM induced lesions by copper supplementation indicates that the lesions are probably due to copper deficiency.

Another group has examined gut pathology in rats receiving approximately 18 mg of TM per kilogram of diet (Fell *et al.*, 1979). These rats also received approximately 3 mg of copper per kilogram of diet. These scientist found gut pathology involving cell apoptosis, edema, and necrosis which they did not attribute to hypocuprosis, although this was not proven. It is probable that a higher copper supplement was required for protection, in view of the finding that all such problems were prevented by adequate copper supplementation (Mills *et al.*, 1981b).

Wilson's disease patients have a huge store of excess copper, so none of the TM toxicities due to copper deficiency are a risk in these patients. Even in the case of the skeletal and enterocyte lesions, since copper administration protected, the Wilson's disease patient with excessive stores of copper should also be protected. Other workers have studied the effect of TM on copper loaded sheep (Gooneratne *et al.*, 1981a). It is well known that sheep are quite susceptible to copper toxicity, usually developing hepatic failure and hemolytic anemia. The studies involved loading sheep dietarily with copper to the point of initiation of hepatic damage, then TM was given intravenously in doses of 50 or 100 mg 2 × weekly for up to 11 weeks.

Five of the 26 sheep died during the study. All deaths were attributed to copper toxicosis based on autopsy results. 3 of the 5 deaths occurred in controls that received copper but not TM. One death occurred after an animal had received only one dose of TM, and another in a animal that had received only 4 doses. It is clear that these 2 animals died from copper toxicity prior to the ability of TM to rescue them. If animals survived the initial onset of copper toxicosis, they were protected from further copper toxicity by TM, even though in some cases copper administration was continued. These animals tolerated up to 22 injections of TM without clinical problems.

Support for the beneficial effect of either IV (Humphries *et al.*, 1986) or subcutaneous (Humphries *et al.*, 1988) TM in protecting sheep against severe hepatic copper toxicity has also been shown. TM not only reduced the amount of hepatic copper, but the actual liver damage. TM was also used prophylactically to prevent copper toxicity in commercial sheep flocks. Over 400 animals have been treated with TM with no adverse side effects (Humphries *et al.*, 1988).

Preliminary work also indicated that TM may be dramatically effective against copper toxicity in the LEC rat model (Suzuki *et al.*, 1993). The genetic defect on these rats has been recently shown to be due to a defect in the Wilson's disease gene (Wu *et al.*, 1994). The rats develop severe liver disease and usually die. TM has been very effective in treating these animals in the late stages of their liver disease.

Mo metabolism in sheep has been studied after the IV injection of ^{99}Mo labeled TM (Mason *et al.*, 1983). There was a rapid plasma disappearance over 15 minutes and then a slow disappearance with a $t_{1/2}$ of about 40 h. The TM was transformed step wise to molybdate and over 90% was excreted in urine compared to 5% in feces. The same group published subsequently on ^{99}Mo and ^{35}S metabolism after IV injection of double labeled TM in sheep (Hynes *et al.*, 1984). Most of the ^{99}Mo and ^{35}S were associated initially with albumin. Displaced or unbound TM was rapidly hydrolyzed to molybdate and sulfate. There was no evidence of an irreversible interaction of either ^{35}S or ^{99}Mo with copper and plasma despite the appearance of a TCA insoluble copper fraction.

It is clear that in the presence of high levels of copper, TM administration results in the accumulation of copper complexed with TM in both the liver and kidneys (Jones *et al.*, 1984; Bremner and Young, 1978). However, there is no evidence of a storage disease associated with this complex. Current theory holds that the complex is disassociated and that the TM is metabolized to oxymolybdates and excreted (Mason *et al.*, 1983). The copper then enters other pathways in the liver. In the presence of high levels of metallothionein it would most likely be taken up by metallothionein. In the kidneys the evidence is that the copper is simply excreted.

Two cases of reversible bone marrow depression have been reported in patients receiving TM for maintenance therapy (Harper and Walshe, 1986). The inventors have seen reversible anemia in seven patients. These patients had a strong response to therapy, and likely ended up with localized, bone marrow, copper deficiency. Since copper is required for heme synthesis, this appears to be a manifestation of over-treatment, at least as far as the bone marrow is concerned. Since TM is such an effective anti-copper agent, during maintenance therapy with TM as in the cases of Harper and Walshe (Harper and Walshe, 1986), it would not be unexpected for over-treatment to occur.

About 37% of TM is Mo. The normal intake of Mo is about 350 μg/day (Seelig, 1972), or the equivalent amount of Mo that would be in about 1.0 mg of TM. Molybdenum seems to be quite well tolerated by the human. Relatively high doses of 5-20 mg/kg/day of Mo (equivalent to the Mo in 1-4 g of TM) were used for 4-11 months in patients with Wilson's disease in a 1957 study, without known toxicity (Bickel *et al.*, 1957). However, it

was not effective, because as pointed out earlier, TM is the active metabolite, and that is formed efficiently from Mo only in ruminants.

C. Other Thiomolybdate Compounds

Other thiomolybdate compounds that have a similar mode of action in free copper reduction include dodecathiodimolybdate, trithiomolybdate, dithiomolybdate and monothiomolybdate. These compounds, like tetrathiomolybdate, form a tripartite complex with copper and protein that renders the copper unavailable, and eventually leads to clearance of the copper-complex.

The synthesis and characterization of a number of thiomolybdate complexes, oxo/thiomolybdate complexes and heterometallic complexes containing iron, molybdenum and sulfur, with or without oxygen, have been described (Coucouvanis, 1998; Coucouvanis *et al.*, 1989; Coucouvanis *et al.*, 1988; Hadjikyriacou and Coucouvanis, 1987; Coucouvanis *et al.*, 1984; Teo *et al.*, 1983; Coucouvanis *et al.*, 1983; Kanatzidis and Coucouvanis, 1983; Coucouvanis *et al.*, 1981; Coucouvanis, 1981; Coucouvanis *et al.*, 1980a, b). These complexes have been shown to bind to copper, and are thus contemplated for use in certain treatment embodiments.

Among the preferred thiomolybdate compounds or complexes are those comprising one, two or three molybdenum atom(s), one molybdenum atom and one iron atom, two molybdenum atoms and one iron atom, and one molybdenum atom and two iron atoms. The structures of three general classes of thiomolybdate compounds, and two of the preferred thiomolybdate compounds, are described in WO 00/13712.

Examples of the thiomolybdate complexes contemplated for use include, but are not limited to, nonathiomolybdate ($[MoS_9]^{2-}$), hexathiodimolybdate ($[Mo_2S_6]^{2-}$), heptathiodimolybdate ($[Mo_2S_7]^{2-}$), octathiodimolybdate ($[Mo_2S_8]^{2-}$), nonathiodimolybdate ($[Mo_2S_9]^{2-}$), decathiodimolybdate ($[Mo_2S_{12}]^{2-}$), undecathiodimolybdate ($[Mo_2S_{11}]^{2-}$), dodecathiodimolybdate ($[Mo_2S_{12}]^{2-}$), the ammonium salt of dodecathiodimolybdate ($(NH_4)_2[Mo_2(S_2)_6]$; Muller *et al.*, 1980; Muller and Krickemeyer, 1990), the ammonium salt of tridecathiotrimolybdate ($(NH_4)_2[Mo_3(S)(S_2)_6]$; Muller and Krickemeyer, 1990), and those

thiomolybdates with an $[FeCl_2S_2MoS_2FeCl_2]^{2-}$, $[S_2MoS_2FeS_2MoS_2]^{3-}$, and $[S_2MoS_2FeCl_2]^{2-}$ core structures.

Additionally, the inventors have discovered that iron gluconate is a source of iron that does not contain halide ions. Its reaction with tetrathiomolybdate in the presence of ammonium hydroxide produces another thiomolybdate compound, the novel ammonium salt of iron octathiodimolybdate ($(NH_4)_3[S_2MoS_2FeS_2MoS_2]$), which is very water soluble. While certain of these compounds may be less potent in reducing copper levels in the body than tetrathiomolybdate, they will nonetheless find utility in particular treatments.

D. Thiomolybdate-Carbohydrate Complexes

The inventors earlier discovered that by forming a complex between thiomolybdate compounds and carbohydrates, such as sucrose, a stabilized form of the thiomolybdate compounds are produced. In these thiomolybdate-carbohydrate complexes, layers of carbohydrate molecules assemble around the thiomolybdate compound in arrays stabilized by hydrogen bonding. These layers serve to protect the thiomolybdate core against oxidation and hydrolysis. Other molecules, such as amino acids, that are capable of hydrogen bonding to the thiomolybdate compounds, were also contemplated for use.

An example of such a stabilized thiomolybdate compound is tetrathiomolybdate stabilized by sucrose. To prepare the sucrose-ammonium tetrathiomolybdate complex, 25 grams of sucrose is dissolved in 20 ml of distilled water. 1 gram of ammonium tetrathiomolybdate is added and the mixture stirred under argon until the TM is solution. The water is then removed from the mixture by flash evaporation under high vacuum.

The term "carbohydrate" embraces a wide variety of chemical compounds having the general formula $(CH_2O)_n$ and encompasses such compounds as monosaccharides, disaccharides, trisaccharides, oligosaccharides, polysaccharides and their aminated, sulfated, acetylated and other derivated forms. Oligosaccharides are chains composed of sugar units, which are also known as monosaccharides. Sugar units can be arranged in any order and linked by their sugar units in any number of different ways. Therefore, the number of different stereoisomeric oligosaccharide chains possible is quite large.

As known in the art, a monosaccharide is a sugar molecule that contains one sugar unit. As used herein, the term "sugar unit" means a monosaccharide. As also known in the art, a disaccharide is a sugar molecule that contains 2 sugar units, a trisaccharide is a sugar molecule that contains 3 sugar units, an oligosaccharide is a sugar molecule that generally contains between about 2 and about 10 sugar units, and a polysaccharide is a sugar molecule that contains greater than 10 sugar units. The sugar units in a di-, tri- and oligosaccharide are all connected by glycosidic linkages. Nonetheless, the term "oligosaccharide" means a sugar molecule that contains at least two sugar units.

"Monosaccharides" will be understood as including, but not being limited to, either the D- or L- isomers of trioses, aldopentoses, aldohexoses, aldotetroses, ketopentoses and ketohexoses. The mentioned compounds may also be in the form of lactones. Examples of an aldopentose include, but are not limited to, ribose, arabinose, xylose and lyose; examples of an aldohexose are allose, altrose, glucose, mannose, gulose, idose, galactose, talose, fucose and rhamnose. Examples of a ketopentose include, but are not limited to, ribulose and xylulose, examples of a tetrose include, but are not limited to, erythrose and threose, and examples of a ketohexose include, but are not limited to, psicose, fructose, sorbose or tagatose.

Examples of a disaccharide are trehalose, maltose, isomaltose, cellobiose, gentiobiose, saccharose, lactose, chitobiose, N,N-diacetylchitobiose, palatinose or sucrose. Examples of trisaccharides are raffinose, panose, melezitose or maltotriose. Examples of oligosaccharides are maltotetraose, maltohexaose or chitoheptaose. The term "carbohydrate" as used herein is also intended to embrace sugar alcohols, *e.g.*, alditols such as mannitol, lactitol, xylitol, glycerol or sorbitol. Examples of polysaccharides include, but are not limited to, polydextrose and maltodextrin.

This stabilization is contemplated to be effective using any carbohydrate as defined herein, at ratios of between about 5 sugar units to about 100 or 200 sugar units or so per thiomolybdate metal center. This range will be understood to include all values within this range, such as ratios of about 10 sugar units per thiomolybdate metal center, about 20 sugar units per thiomolybdate metal center, about 25 sugar units per thiomolybdate metal center, about 30 sugar units per thiomolybdate metal center, about 40 sugar units per thiomolybdate

metal center, about 50 sugar units per thiomolybdate metal center, about 60 sugar units per thiomolybdate metal center, about 70 sugar units per thiomolybdate metal center, about 75 sugar units per thiomolybdate metal center, about 80 sugar units per thiomolybdate metal center, about 90 sugar units per thiomolybdate metal center, about 110 sugar units per thiomolybdate metal center, about 125 sugar units per thiomolybdate metal center, about 150 sugar units per thiomolybdate metal center, about 175 sugar units per thiomolybdate metal center or about 190 sugar units per thiomolybdate metal center.

III. Stabilized Thiomolybdate Compounds

Thiomolybdate complexes are sensitive to oxidation when exposed to air. This imposes certain practical obstacles to the ready formulation and use of such compounds in the clinic. Therefore, in order to progress the state of the art in the clinical treatment of angiogenic diseases using copper complexation, the inventors developed a range of improved thiomolybdate compounds with enhanced stability. The resultant compounds are easier to formulate into pharmaceutically acceptable vehicles, and when so fabricated, have an improved shelf life.

In designing and selecting the new molecules, it was important not to obtain stability at the price of reduced solubility or efficacy. Those of ordinary skill in the art understand that the interrelationship between physical, chemical and biological properties of a given class of compounds is such that until a lead compound has been made and tested, the presence of the desired properties, without any new drawbacks, cannot be predicted. The present invention therefore provides a series of new compounds in the context of demonstrated improvements in stability, without any loss of solubility, anti-copper properties or therapeutic activity in controlled studies. The compounds, pharmaceutical formulations and kits provided by the invention therefore have advantages in the ease of preparation and handling, and the increased shelf life, without any downside in their therapeutic use.

The new compounds are based upon alkylammonium thiomolybdate compounds, a preferred example of which is tetrapropylammonium tetrathiomolybdate (TP-TM). This terminology is used succinctly throughout the present application, and those of ordinary skill in the art will understand that more accurate name to be "di-tetrapropylammonium-tetrathiomolybdate", $[N(C_3H_7)_4]^+MoS_4^{2-}[N(C_3H_7)_4]^+$. Analogous derivatives of

tetrathiotungstate compounds may also be used in the invention, wherein tungsten is employed in place of molybdenum.

This new salt, and the related alkylammonium thiomolybdate compounds, stabilize the thiomolybdate center of the molecule, whilst retaining solubility, low toxicity and anticopper properties. With the ammonium salt of tetrathiomolybdate (AmmTM or TM) used in the inventors' early studies, oxygen replaces the sulfur during exposure to air, rendering the compound inactive in terms of its important anticopper properties. This process was catalyzed further by any moisture in the air.

In the new family of molecules, the alkyl groups surround the thiomolybdate core, protecting it from air and moisture. In preliminary tests, stability in moist heated air (which increases loss of activity in AmmTM about 10-fold over ambient conditions) is evident for at least 7 days. Stability over a 2 month period was subsequently demonstrated. The half life of TP-TM, for example, in open Petri dishes at room temperature is about 180 days, as opposed to only about 40 days for AmmTM.

However, the compounds retain sufficient solubility for pharmaceutical purposes, as exemplified by TP-TM, which is soluble to at least 1 mg/ml of water, which is more than adequate. In an *in vitro* assay, the new compounds such as TP-TM show the standard copper-albumin-TM tripartite binding of the parent AmmTM compound that is predictive of biological anticopper activity. The difference in stability with maintained solubility and copper binding properties makes TP-TM a greatly improved drug over AmmTM.

However, the present invention does not rely solely on such *in vitro* data, but demonstrates the effectiveness of the new compounds, and their lack of toxicity, *in vivo*. In studies in art-accepted *in vivo* models, the effectiveness of TP-TM, representative of the new compounds, was indistinguishable from that of AmmTM in inhibiting tumor growth.

The inventors also present *in vivo* data showing the safety of the new class of compounds, particularly TP-TM. Although tetramethylammonium, and to a lesser extent, tetraethylammonium, which will be released from the related compounds, may have some CNS ganglionic blocking activity, this can be managed by the attending physician,

particularly in light of the safety margin established in the following TP-TM study. The release of the tetrapropylammonium moieties from TP-TM was represented in an *in vivo* study by the simple administration of tetrapropylammonium chloride. At doses up to 466-fold higher than would be required for anti-tumor effects in humans, all animals were alive and healthy after 58 doses. These data therefore show that the salt released from TP-TM is non-toxic at doses many fold higher than could possibly be required for copper-reducing therapy with this compound in humans.

The new compounds are therefore stable, soluble, therapeutically effective and substantially non-toxic, and represent a surprising improvement over the existing AmmTM and related, first generation compounds.

IV. Monitoring Copper Levels

The serum ceruloplasmin, which is directly dependent upon liver copper status, is an accurate indicator of copper status. The dose of TM used in previous studies in rodents would average about 0.5 mg/day in rats of average weight. The inventors' studies in mice and rats, and the inventors' extensive experience in humans with Wilson's disease and in humans with cancer, indicate that 4 times that dose is required in rats to reduce serum ceruloplasmin to about 10% of normal which is the optimal criterion for low copper status in rodents. These concepts of optimal monitoring of Cu status and TM dosing were subsequently incorporated in the animal studies of TM described in Example 2.

Based on the studies herein, and the inventors experience with TM, reduction in the level of ceruloplasmin (Cp) to between about 40% and about 10% of the baseline value prior to treatment will result in at least some level of beneficial clinical anti-angiogenic effect. However, a reduction of Cp to a level of about 20% of the baseline value prior to treatment is preferred in most clinical indications. The data presented herein concerning TP-TM show that TP-TM is at least as effective as TM in inhibiting tumor growth *in vivo* (FIG. 5). Therefore, the volume of data available on TM will be readily applicable to the clinical use of TP-TM and related compounds.

V. Combination Therapies

The methods of the present invention may be combined with any other methods generally employed in the treatment of the particular disease or disorder that the patient exhibits. For example, in connection with the treatment of solid tumors, the methods of the present invention may be used in combination with classical approaches, such as surgery, radiotherapy and the like. So long as a particular therapeutic approach is not known to be detrimental in itself, or counteracts the effectiveness of the present therapy, its combination with the invention is contemplated. When one or more agents are used in combination with the more stable thiomolybdate compounds, such as TP-TM, there is no requirement for the combined results to be additive of the effects observed when each treatment is conducted separately, although this is evidently desirable, and there is no particular requirement for the combined treatment to exhibit synergistic effects, although this is certainly possible and advantageous.

In terms of surgery, concurrent surgery during copper deficiency is not preferred since blood vessel growth is required for wound healing. However, as copper can be repleted within about 24 hours of discontinuing therapy, any surgical intervention may be practiced after copper repletion. The invention can then be reinstated after wound healing has been established, typically about 1-2 weeks following surgery. In connection with radiotherapy, any mechanism for inducing DNA damage locally within tumor cells is contemplated, such as γ-irradiation, X-rays, UV-irradiation, microwaves and even electronic emissions and the like. The directed delivery of radioisotopes to tumor cells is also contemplated, and this may be used in connection with a targeting antibody or other targeting means.

Cytokine therapy also has proven to be an effective partner for combined therapeutic regimens. Various cytokines may be employed in such combined approaches. Examples of cytokines include IL-1α IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF-β, GM-CSF, M-CSF, G-CSF, TNFα, TNFβ, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β, IFN-γ. Cytokines are administered according to standard regimens, consistent with clinical indications such as the condition of the patient and relative toxicity of the cytokine.

A. Copper Chelating Agents

1. Zinc

The inventors contemplate the use of zinc following achievement of a low-copper status in cancer patients in order to maintain a moderate copper-deficient state for the long-term. The inventors have developed zinc as an anti-copper agent for Wilson's disease, and it was approved for this purpose in January 1997 by the United States Food and Drug Administration (FDA). Zinc acts by inducing metallothionein in the intestinal mucosal cell, thereby blocking copper absorption (Brewer and Yuzbasiyan-Gurkan, 1992a). Zinc too, is extremely safe. In studies now reaching 200 Wilson's disease patients, zinc in doses of 150 mg daily has produced no toxicity at all (Brewer and Yuzbasiyan-Gurkan, 1992a).

Zinc compounds, such as zinc acetate, are thus being used for the comprehensive treatment of Wilson's disease including initial treatment (Hoogenraad *et al.*, 1978; Hoogenraad *et al.*, 1979; Hoogenraad *et al.*, 1987). However, zinc is not ideal for initial therapy by itself, largely because it is rather slow acting. Thus, it takes approximately two weeks to achieve intestinal metallothionein induction and a negative copper balance (Yuzbasiyan-Gurkan *et al.*, 1992). At the two week point, zinc immediately reverses the +0.54 mg daily (positive) copper balance these patients average, but the negative copper balance induced is rather modest, averaging -0.35 mg daily (negative) copper balance (Brewer *et al.*, 1990; Brewer *et al.*, 1993b). Due to this low rate of copper removal, it takes as long as six months of zinc therapy to bring urine copper and nonceruloplasmin plasma copper (the potentially toxic copper measured in the blood), down to subtoxic levels.

Nonetheless, the use of zinc as a maintenance therapy after achieving low-copper status remains a preferred aspect of the invention. Due to the fact that zinc compounds are relatively inexpensive and easy to prepare, and their widespread availability, even the use of zinc compounds to achieve initial low-copper status remains an aspect of the overall invention.

2. Penicillamine

Penicillamine is the drug that has been used the most, and is the best known. However, it should be the last choice for initial treatment of neurological patients because of the very high risk of making them neurologically worse (Brewer *et al.*, 1987a; Glass *et al.*,

1990; Brewer *et al.*, 1994a). Another problem with penicillamine is that about a quarter to a third of patients develop an initial hypersensitivity syndrome, requiring significant interventions, such as temporarily stopping the drug and restarting it at a lower dose, usually with concurrent cortico-steroid administration. This is a somewhat frightening experience for patients who are already ill, and prevents the attending physician in the inventors' study from being blinded. Finally, there is a long list of other side effects that can occur with penicillamine during the first few weeks of therapy. These include bone marrow depression, proteinuria, and auto-immune disorders.

3. Trientine

Trientine acts by chelation and urinary excretion of copper (Walshe, 1982). A therapeutic dose (1,000-2,000 mg/day) usually produces only about half as much cupruresis as a similar dose of penicillamine. Nonetheless, trientine is capable of an initial production of a several mg negative copper balance, much greater than zinc. Typically, this 4-5 mg cupruresis decreases during the first few weeks of therapy to a more modest, but still substantial, 2-3 mg. Ingestion of copper is about 1 mg/day, with obligatory, non-urine losses of about 0.5 mg. Thus a cupruresis of 2-3 mg produces a negative copper balance of 1.5 to 2.5 mg/day.

Trientine is officially approved for use in patients intolerant of penicillamine therapy. Because of this, and because it was introduced much later than penicillamine, it has not been used and reported on very extensively. It has not had a formal toxicity study. It appears to have substantially less risk of side effects then penicillamine. An initial hypersensitivity problem has not been reported. It does cause proteinuria, after several weeks of use in about 20% of patients. It can also occasionally produce bone marrow depression and autoimmune abnormalities, although the latter is usually after prolonged use.

So far, trientine has not been reported to cause initial worsening in neurological patients, but its sole use in this type of patient is probably very limited. Anecdotally, the inventors have received patients in transfer who worsened on penicillamine, were switched briefly to trientine, and when they became worse (or failed to improve) were transferred to the inventors for TM therapy. In these kinds of patients it is impossible to know if trientine played any role in worsening. Theoretically, it could, because as with penicillamine, it

mobilizes copper, producing a higher blood level to achieve urinary excretion. But whether this increased level of blood copper translates into increased brain levels, and increased neurotoxicity, is unknown.

B. Chemotherapeutic Combinations and Treatment

Irrespective of the mechanisms by which enhanced tumor destruction is achieved, the combined treatment aspects of the present invention have evident utility in the effective treatment of disease. To use the present invention in combination with the administration of a chemotherapeutic agent, one would simply administer to an animal a more stable thiomolybdate compound, such as TP-TM, in combination with the chemotherapeutic agent in a manner effective to result in their combined anti-tumor actions within the animal. These agents would therefore be provided in an amount effective and for a period of time effective to result in their combined presence within the tumor vasculature and their combined actions in the tumor environment. To achieve this goal, the more stable thiomolybdate compound, such as TP-TM, and the chemotherapeutic agent may be administered to the animal simultaneously, either in a single composition or as two distinct compositions using different administration routes.

Alternatively, treatment with a more stable thiomolybdate compound, such as TP-TM, may precede or follow the chemotherapeutic agent treatment by intervals ranging from minutes to weeks. In embodiments where the chemotherapeutic factor and more stable thiomolybdate compound are applied separately to the animal, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the chemotherapeutic agent and more stable thiomolybdate compound would still be able to exert an advantageously combined effect on the tumor. In such instances, it is contemplated that one would contact the tumor with both agents within about 5 minutes to about one week of each other and, more preferably, within about 12-72 hours of each other, with a delay time of only about 12-48 hours being most preferred.

However, in some situations, it may be desirable to extend the time period for treatment significantly, where several days (2, 3, 4, 5, 6 or 7) or even several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations. Additionally, a preferred embodiment of the present invention is to administer the more stable thiomolybdate

compound, such as TP-TM, between rounds of chemotherapy, or in a maintenance regimen after chemotherapy. Thus, it also is conceivable that more than one administration of either the more stable thiomolybdate compound, such as TP-TM, and/or the chemotherapeutic agent will be desired. To achieve tumor regression, both agents are delivered in a combined amount effective to inhibit its growth, irrespective of the times for administration.

A variety of chemotherapeutic agents are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated as exemplary include, *e.g.*, etoposide (VP-16), adriamycin, 5-fluorouracil (5FU), camptothecin, actinomycin-D, mitomycin C, carbaplatin, paclitaxel, docetaxel and even hydrogen peroxide. As will be understood by those of ordinary skill in the art, the appropriate doses of chemotherapeutic agents will be generally around those already employed in clinical therapies wherein the chemotherapeutics are administered alone or in combination with other chemotherapeutics.

Further useful agents include compounds that interfere with DNA replication, mitosis and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in a clinical setting for the treatment of neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m^2 at 21 day intervals for adriamycin, to 35-50 mg/m^2 for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of polynucleotide precursors may also be used. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU) are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Exemplary chemotherapeutic agents that are useful in connection with combined therapy are listed in Table 1. Each of the agents listed therein are exemplary and by no means limiting. The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th

Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Table 1

Chemotherapeutic Agents Useful In Neoplastic Disease

CLASS	TYPE OF AGENT	NONPROPRIETARY NAMES (OTHER NAMES)	DISEASE
Alkylating Agents	Nitrogen Mustards	Mechlorethamine (HN_2)	Hodgkin's disease, non-Hodgkin's lymphomas
		Cyclophosphamide Ifosfamide	Acute and chronic lymphocytic leukemias, Hodgkin's disease, non-Hodgkin's lymphomas, multiple myeloma, neuroblastoma, breast, ovary, lung, Wilms' tumor, cervix, testis, soft-tissue sarcomas
		Melphalan (L-sarcolysin)	Multiple myeloma, breast, ovary
		Chlorambucil	Chronic lymphocytic leukemia, primary macroglobulinemia, Hodgkin's disease, non-Hodgkin's lymphomas
	Ethylenimenes and Methylmelamines	Hexamethyl-melamine	Ovary
		Thiotepa	Bladder, breast, ovary
	Alkyl Sulfonates	Busulfan	Chronic granulocytic leukemia
	Nitrosoureas	Carmustine (BCNU)	Hodgkin's disease, non-Hodgkin's lymphomas, primary brain tumors, multiple myeloma, malignant melanoma
		Lomustine (CCNU)	Hodgkin's disease, non-Hodgkin's lymphomas, primary brain tumors, small-cell lung
		Semustine (methyl-CCNU)	Primary brain tumors, stomach, colon
		Streptozocin (streptozotocin)	Malignant pancreatic insulinoma, malignant carcinoid
	Triazines	Dacarbazine, DTIC; dimethyltriazenoimid-azolecarboxamide	Malignant melanoma, Hodgkin's disease, soft-tissue sarcomas
Anti-metabolites	Folic Acid Analogs	Methotrexate (amethopterin)	Acute lymphocytic leukemia, choriocarcinoma, mycosis fungoides, breast, head and neck, lung, osteogenic sarcoma
	Pyrimidine Analogs	Fluouracil (5-fluorouracil; 5-FU) Floxuridine (fluorode-oxyuridine; FUdR)	Breast, colon, stomach, pancreas, ovary, head and neck, urinary bladder, premalignant skin lesions (topical)
		Cytarabine (cytosine arabinoside)	Acute granulocytic and acute lymphocytic leukemias
	Purine Analogs and Related Inhibitors	Mercaptopurine 6-mercaptopurine 6-MP	Acute lymphocytic, acute granulocytic and chronic granulocytic leukemias
		Thioguanine 6-thioguanine; TG	Acute granulocytic, acute lymphocytic and chronic granulocytic leukemias
		Pentostatin 2-deoxycoformycin	Hairy cell leukemia, mycosis fungoides, chronic lymphocytic leukemia

CLASS	TYPE OF AGENT	NONPROPRIETARY NAMES (OTHER NAMES)	DISEASE
Natural Products	Vinca Alkaloids	Vinblastine (VLB)	Hodgkin's disease, non-Hodgkin's lymphomas, breast, testis
		Vincristine	Acute lymphocytic leukemia, neuroblastoma, Wilms' tumor, rhabdomyosarcoma, Hodgkin's disease, non-Hodgkin's lymphomas, small-cell lung
	Epipodo-phyllotoxins	Etoposide Tertiposide	Testis, small-cell lung and other lung, breast, Hodgkin's disease, non-Hodgkin's lymphomas, acute granulocytic leukemia, Kaposi's sarcoma
	Antibiotics	Dactinomycin (actinomycin D)	Choriocarcinoma, Wilms' tumor, rhabdomyosarcoma, testis, Kaposi's sarcoma
		Daunorubicin (daunomycin; rubidomycin)	Acute granulocytic and acute lymphocytic leukemias
		Doxorubicin	Soft-tissue, osteogenic and other sarcomas; Hodgkin's disease, non-Hodgkin's lymphomas, acute leukemias, breast, genitourinary, thyroid, lung, stomach, neuroblastoma
		Bleomycin	Testis, head and neck, skin, esophagus, lung and genitourinary tract; Hodgkin's disease, non-Hodgkin's lymphomas
		Plicamycin (mithramycin)	Testis, malignant hypercalcemia
		Mitomycin (mitomycin C)	Stomach, cervix, colon, breast, pancreas, bladder, head and neck
	Enzymes	L-Asparaginase	Acute lymphocytic leukemia
	Biological Response Modifiers	Interferon alfa	Hairy cell leukemia., Kaposi's sarcoma, melanoma, carcinoid, renal cell, ovary, bladder, non-Hodgkin's lymphomas, mycosis fungoides, multiple myeloma, chronic granulocytic leukemia
Other Agents	Platinum Coordination Complexes	Cisplatin (*cis*-DDP) Carboplatin	Testis, ovary, bladder, head and neck, lung, thyroid, cervix, endometrium, neuroblastoma, osteogenic sarcoma
	Anthracenedione	Mitoxantrone	Acute granulocytic leukemia, breast
	Substituted Urea	Hydroxyurea	Chronic granulocytic leukemia, polycythemia vera, essental thrombocytosis, malignant melanoma
	Methyl Hydrazine Derivative	Procarbazine (N-methylhydrazine, MIH)	Hodgkin's disease
	Adrenocortical Suppressant	Mitotane (*o,p'*-DDD)	Adrenal cortex
		Aminoglutethimide	Breast
Hormones and Antagonists	Adrenocorticosteroids	Prednisone (several other equivalent preparations available)	Acute and chronic lymphocytic leukemias, non-Hodgkin's lymphomas, Hodgkin's disease, breast

CLASS	TYPE OF AGENT	NONPROPRIETARY NAMES (OTHER NAMES)	DISEASE
	Progestins	Hydroxyprogesterone caproate Medroxyprogesterone acetate Megestrol acetate	Endometrium, breast
	Estrogens	Diethylstilbestrol Ethinyl estradiol (other preparations available)	Breast, prostate
	Antiestrogen	Tamoxifen	Breast
	Androgens	Testosterone propionate Fluoxymesterone (other preparations available)	Breast
	Antiandrogen	Flutamide	Prostate
	Gonadotropin-releasing hormone analog	Leuprolide	Prostate

C. Anti-Angiogenics

The term "angiogenesis" refers to the generation of new blood vessels, generally into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonic development and formation of the corpus luteum, endometrium and placenta.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

The more stable thiomolybdate compounds of the invention, such as TP-TM, may be used in combination with any one or more other anti-angiogenic therapies. Combinations with agents that inhibit VEGF are particularly included, such as neutralizing antibodies, soluble receptor constructs, tyrosine kinase inhibitors, antisense strategies, RNA aptamers

and ribozymes against VEGF or VEGF receptors. Variants of VEGF with antagonistic properties may also be employed, as described in WO 98/16551.

In general, the anti-angiogenic therapies may be based upon the provision of an anti-angiogenic agent or the inhibition of an angiogenic agent. For example, antibodies to angiogenin may be employed, as described in U.S. Patent No. 5,520,914, specifically incorporated herein by reference. In that FGF is connected with angiogenesis, FGF inhibitors may also be used. Certain examples are the compounds having N-acetylglucosamine alternating in sequence with 2-O-sulfated uronic acid as their major repeating units, including glycosaminoglycans, such as archaran sulfate. Such compounds are described in U.S. Patent No. 6,028,061, specifically incorporated herein by reference, and may be used in combination herewith.

Numerous tyrosine kinase inhibitors useful for the treatment of angiogenesis, as manifest in various diseases states, are now known. These include, for example, the 4-aminopyrrolo[2,3-d]pyrimidines of U.S. Patent No. 5,639,757, specifically incorporated herein by reference, which may also be used in combination with the present invention. Further examples of organic molecules capable of modulating tyrosine kinase signal transduction via the VEGFR2 receptor are the quinazoline compounds and compositions of U.S. Patent No. 5,792,771, which is specifically incorporated herein by reference for the purpose of describing further combinations for use with the present invention in the treatment of angiogenic diseases.

Compounds of other chemical classes have also been shown to inhibit angiogenesis and may be used in combination with the present invention. For example, steroids such as the angiostatic 4,9(11)-steroids and C21-oxygenated steroids, as described in U.S. Patent No. 5,972,922, specifically incorporated herein by reference, may be employed in combined therapy. U.S. Patent No. 5,712,291 and 5,593,990, each specifically incorporated herein by reference, describe thalidomide and related compounds, precursors, analogs, metabolites and hydrolysis products, which may also be used in combination with the present invention to inhibit angiogenesis. The compounds in U.S. Patent No. 5,712,291 and 5,593,990 can be administered orally. Further exemplary anti-angiogenic agents that are useful in connection

with combined therapy are listed in Table 2. Each of the agents listed therein are exemplary and by no means limiting.

Table 2

Inhibitors and Negative Regulators of Angiogenesis

Angiostatin
Endostatin
16kDa prolactin fragment
Laminin peptides
Fibronectin peptides
Tissue metalloproteinase inhibitors (TIMP 1, 2, 3, 4)
Plasminogen activator inhibitors (PAI-1, -2)
Tumor necrosis factor α (high dose, *in vitro*)
TGF-β1
Interferons (IFN-α, -β, γ)
ELR- CXC Chemokines: IL-12; SDF-1; MIG; Platelet factor 4 (PF-4); IP-10
Thrombospondin (TSP)
SPARC
2-Methoxyoestradiol
Proliferin-related protein
Suramin
Thalidomide
Cortisone
Linomide
Fumagillin (AGM-1470; TNP-470)
Tamoxifen
Korean mistletoe extract (*Viscum album coloratum*)
Retinoids
CM101
Dexamethasone
Leukemia inhibitory factor (LIF)

Certain components for use in inhibiting angiogenesis in combination with the more stable thiomolybdate compounds of the invention, such as TP-TM, are angiostatin, endostatin, vasculostatin, canstatin and maspin. Angiostatin is disclosed in U.S. Patents 5,776,704; 5,639,725 and 5,733,876, each incorporated herein by reference. Angiostatin is a

protein having a molecular weight of between about 38 kD and about 45 kD, as determined by reducing polyacrylamide gel electrophoresis, which contains approximately Kringle regions 1 through 4 of a plasminogen molecule. Angiostatin generally has an amino acid sequence substantially similar to that of a fragment of murine plasminogen beginning at amino acid number 98 of an intact murine plasminogen molecule.

The amino acid sequence of angiostatin varies slightly between species. For example, in human angiostatin, the amino acid sequence is substantially similar to the sequence of the above described murine plasminogen fragment, although an active human angiostatin sequence may start at either amino acid number 97 or 99 of an intact human plasminogen amino acid sequence. Further, human plasminogen may be used, as it has similar anti-angiogenic activity, as shown in a mouse tumor model.

Angiostatin and endostatin have become the focus of intense study, as they are the first angiogenesis inhibitors that have demonstrated the ability to not only inhibit tumor growth but also cause tumor regressions in mice. There are multiple proteases that have been shown to produce angiostatin from plasminogen including elastase, macrophage metalloelastase (MME), matrilysin (MMP-7), and 92 kDa gelatinase B/type IV collagenase (MMP-9).

MME can produce angiostatin from plasminogen in tumors and granulocyte-macrophage colony-stimulating factor (GMCSF) upregulates the expression of MME by macrophages inducing the production of angiostatin. The role of MME in angiostatin generation is supported by the finding that MME is in fact expressed in clinical samples of hepatocellular carcinomas from patients. Another protease thought to be capable of producing angiostatin is stromelysin-1 (MMP-3). MMP-3 has been shown to produce angiostatin-like fragments from plasminogen *in vitro*. The mechanism of action for angiostatin is currently unclear, it is hypothesized that it binds to an unidentified cell surface receptor on endothelial cells inducing endothelial cell to undergo programmed cell death or mitotic arrest.

Endostatin appears to be an even more powerful anti-angiogenesis and anti-tumor agent. Endostatin is effective at causing regressions in a number of tumor models in mice.

Tumors do not develop resistance to endostatin and, after multiple cycles of treatment, tumors enter a dormant state during which they do not increase in volume. In this dormant state, the percentage of tumor cells undergoing apoptosis was increased, yielding a population that essentially stays the same size.

U.S. Patent No. 5,854,205, to Folkman and O'Reilly, specifically incorporated herein by reference, concerns endostatin and its use as an inhibitor of endothelial cell proliferation and angiogenesis. The endostatin protein corresponds to a C-terminal fragment of collagen type XVIII, and the protein can be isolated from a variety of sources. U.S. Patent No. 5,854,205 also teaches that endostatin can have an amino acid sequence of a fragment of collagen type XVIII, a collasen type XV, or BOVMPE 1 pregastric esterase. Combinations of endostatin with other anti-angiogenic proteins, particularly angiostatin, are also described by U.S. Patent No. 5,854,205, such that the combined compositions are capable of effectively regressing the mass of an angiogenesis-dependent tumor. Vasculostatin, canstatin and maspin are also suitable for combination with the present invention.

Certain anti-angiogenic therapies have already been shown to cause tumor regressions, including the bacterial polysaccharide CM101 and the antibody LM609. CM101 is a bacterial polysaccharide that has been well characterized in its ability to induce neovascular inflammation in tumors. CM101 binds to and cross-links receptors expressed on dedifferentiated endothelium that stimulates the activation of the complement system. It also initiates a cytokine-driven inflammatory response that selectively targets the tumor. It is a uniquely antipathoangiogenic agent that downregulates the expression VEGF and its receptors. CM101 is currently in clinical trials as an anti-cancer drug, and can be used in combination herewith.

Thrombospondin (TSP-1) and platelet factor 4 (PF4) may also be used in combination with the present invention. These are both angiogenesis inhibitors that associate with heparin and are found in platelet α-granules. TSP-1 is a large 450kDa multi-domain glycoprotein that is constituent of the extracellular matrix. TSP-1 binds to many of the proteoglycan molecules found in the extracellular matrix including, HSPGs, fibronectin, laminin, and different types of collagen. TSP-1 inhibits endothelial cell migration and proliferation *in vitro* and angiogenesis *in vivo*. TSP-1 can also suppress the malignant phenotype and

tumorigenesis of transformed endothelial cells. The tumor suppressor gene p53 has been shown to directly regulate the expression of TSP-1 such that, loss of p53 activity causes a dramatic reduction in TSP-1 production and a concomitant increase in tumor initiated angiogenesis.

PF4 is a 70aa protein that is member of the CXC ELR- family of chemokines that is able to potently inhibit endothelial cell proliferation *in vitro* and angiogenesis *in vivo*. PF4 administered intratumorally or delivered by an adenoviral vector is able to cause an inhibition of tumor growth.

Interferons and metalloproteinase inhibitors are two other classes of naturally occurring angiogenic inhibitors that can be combined with the present invention. The anti-endothelial activity of the interferons has been known since the early 1980s, however, the mechanism of inhibition is still unclear. It is known that they can inhibit endothelial cell migration and that they do have some anti-angiogenic activity *in vivo* that is possibly mediated by an ability to inhibit the production of angiogenic promoters by tumor cells. Vascular tumors in particular are sensitive to interferon, for example, proliferating hemangiomas can be successfully treated with IFNα.

Tissue inhibitors of metalloproteinases (TIMPs) are a family of naturally occurring inhibitors of matrix metalloproteases (MMPs) that can also inhibit angiogenesis and can be used in combined treatment protocols. MMPs play a key role in the angiogenic process as they degrade the matrix through which endothelial cells and fibroblasts migrate when extending or remodeling the vascular network. In fact, one member of the MMPs, MMP-2, has been shown to associate with activated endothelium through the integrin $\alpha v\beta 3$ presumably for this purpose. If this interaction is disrupted by a fragment of MMP-2, then angiogenesis is downregulated and in tumors growth is inhibited.

There are a number of pharmacological agents that inhibit angiogenesis, any one or more of which may be used in combination with the present invention. These include AGM-1470/TNP-470, thalidomide, and carboxyamidotriazole (CAI). Fumagillin was found to be a potent inhibitor of angiogenesis in 1990, and since then the synthetic analogues of fumagillin, AGM-1470 and TNP-470 have been developed. Both of these drugs inhibit endothelial cell

proliferation *in vitro* and angiogenesis *in vivo*. TNP-470 has been studied extensively in human clinical trials with data suggesting that long-term administration is optimal.

Thalidomide was originally used as a sedative but was found to be a potent teratogen and was discontinued. In 1994 it was found that thalidomide is an angiogenesis inhibitor. Thalidomide is currently in clinical trials as an anti-cancer agent as well as a treatment of vascular eye diseases.

CAI is a small molecular weight synthetic inhibitor of angiogenesis that acts as a calcium channel blocker that prevents actin reorganization, endothelial cell migration and spreading on collagen IV. CAI inhibits neovascularization at physiological attainable concentrations and is well tolerated orally by cancer patients. Clinical trials with CAI have yielded disease stabilization in 49 % of cancer patients having progressive disease before treatment.

Cortisone in the presence of heparin or heparin fragments was shown to inhibit tumor growth in mice by blocking endothelial cell proliferation. The mechanism involved in the additive inhibitory effect of the steroid and heparin is unclear although it is thought that the heparin may increase the uptake of the steroid by endothelial cells. The mixture has been shown to increase the dissolution of the basement membrane underneath newly formed capillaries and this is also a possible explanation for the additive angiostatic effect. Heparin-cortisol conjugates also have potent angiostatic and anti-tumor effects activity *in vivo*.

Further specific angiogenesis inhibitors, including, but not limited to, Anti-Invasive Factor, retinoic acids and paclitaxel (U.S. Patent No. 5,716,981; incorporated herein by reference); AGM-1470 (Ingber *et al.*, 1990; incorporated herein by reference); shark cartilage extract (U.S. Patent No. 5,618,925; incorporated herein by reference); anionic polyamide or polyurea oligomers (U.S. Patent No. 5,593,664; incorporated herein by reference); oxindole derivatives (U.S. Patent No. 5,576,330; incorporated herein by reference); estradiol derivatives (U.S. Patent No. 5,504,074; incorporated herein by reference); and thiazolopyrimidine derivatives (U.S. Patent No. 5,599,813; incorporated herein by reference) are also contemplated for use as anti-angiogenic compositions for the combined uses of the present invention.

Compositions comprising an antagonist of an $\alpha_v\beta_3$ integrin may also be used to inhibit angiogenesis in combination with the present invention. As disclosed in U.S. Patent No. 5,766,591 (incorporated herein by reference), RGD-containing polypeptides and salts thereof, including cyclic polypeptides, are suitable examples of $\alpha_v\beta_3$ integrin antagonists.

The antibody LM609 against the $\alpha_v\beta_3$ integrin also induces tumors regressions. Integrin $\alpha_v\beta_3$ antagonists, such as LM609, induce apoptosis of angiogenic endothelial cells leaving the quiescent blood vessels unaffected. LM609 or other $\alpha_v\beta_3$ antagonists may also work by inhibiting the interaction of $\alpha_v\beta_3$ and MMP-2, a proteolytic enzyme thought to play an important role in migration of endothelial cells and fibroblasts. U.S. Patent No. 5,753,230 is specifically incorporated herein by reference to describe antibodies against $\alpha_v\beta_3$ (vitronectin $\alpha_v\beta_3$) for combined with the present invention for inhibiting angiogenesis.

Apoptosis of the angiogenic endothelium in this case may have a cascade effect on the rest of the vascular network. Inhibiting the tumor vascular network from completely responding to the tumor's signal to expand may, in fact, initiate the partial or full collapse of the network resulting in tumor cell death and loss of tumor volume. It is possible that endostatin and angiostatin function in a similar fashion. The fact that LM609 does not affect quiescent vessels but is able to cause tumor regressions suggests strongly that not all blood vessels in a tumor need to be targeted for treatment in order to obtain an anti-tumor effect.

Other methods of therapeutic intervention based upon altering signaling through the Tie2 receptor can also be used in combination with the present invention, such as using a soluble Tie2 receptor capable of blocking Tie2 activation. Delivery of such a construct using recombinant adenoviral gene therapy has been shown to be effective in treating cancer and reducing metastases.

D. Apoptosis-Inducing Agents

The more stable thiomolybdate compounds of the invention, such as TP-TM, may also be combined with treatment methods that induce apoptosis in any cells within the tumor, including tumor cells and tumor vascular endothelial cells. Although many anti-cancer

agents may have, as part of their mechanism of action, an apoptosis-inducing effect, certain agents have been discovered, designed or selected with this as a primary mechanism, as described below.

A number of oncogenes have been described that inhibit apoptosis, or programmed cell death. Exemplary oncogenes in this category include, but are not limited to, bcr-abl, bcl-2 (distinct from bcl-1, cyclin D1; GenBank accession numbers M14745, X06487; U.S. Patent No. 5,650,491; and 5,539,094; each incorporated herein by reference) and family members including Bcl-xl, Mcl-1, Bak, A1, A20. Overexpression of bcl-2 was first discovered in T cell lymphomas. bcl-2 functions as an oncogene by binding and inactivating Bax, a protein in the apoptotic pathway. Inhibition of bcl-2 function prevents inactivation of Bax, and allows the apoptotic pathway to proceed. Thus, inhibition of this class of oncogenes, *e.g.*, using antisense nucleotide sequences, is contemplated for use in the present invention in aspects wherein enhancement of apoptosis is desired (U.S. Patent No. 5,650,491; 5,539,094; and 5,583,034; each incorporated herein by reference).

Many forms of cancer have reports of mutations in tumor suppressor genes, such as p53. Inactivation of p53 results in a failure to promote apoptosis. With this failure, cancer cells progress in tumorigenesis, rather than become destined for cell death. Thus, provision of tumor suppressors are also contemplated for use in the present invention to stimulate cell death. Exemplary tumor suppressors include, but are not limited to, p53, Retinoblastoma gene (Rb), Wilm's tumor (WT1), bax alpha, interleukin-1b-converting enzyme and family, MEN-1 gene, neurofibromatosis, type 1 (NF1), cdk inhibitor p16, colorectal cancer gene (DCC), familial adenomatosis polyposis gene (FAP), multiple tumor suppressor gene (MTS-1), BRCA1 and BRCA2.

Preferred for use are the p53 (U.S. Patent No. 5,747,469; 5,677,178; and 5,756,455; each incorporated herein by reference), Retinoblastoma, BRCA1 (U.S. Patent No. 5,750,400; 5,654,155; 5,710,001; 5,756,294; 5,709,999; 5,693,473; 5,753,441; 5,622,829; and 5,747,282; each incorporated herein by reference), MEN-1 (GenBank accession number U93236) and adenovirus E1A (U.S. Patent No. 5,776,743; incorporated herein by reference) genes.

Other compositions that may be used include genes encoding the tumor necrosis factor related apoptosis inducing ligand termed TRAIL, and the TRAIL polypeptide (U.S. Patent No. 5,763,223; incorporated herein by reference); the 24 kDa apoptosis-associated protease of U.S. Patent No. 5,605,826 (incorporated herein by reference); Fas-associated factor 1, FAF1 (U.S. Patent No. 5,750,653; incorporated herein by reference). Also contemplated for use in these aspects of the present invention is the provision of interleukin-1β-converting enzyme and family members, which are also reported to stimulate apoptosis.

Compounds such as carbostyril derivatives (U.S. Patent No. 5,672,603; and 5,464,833; each incorporated herein by reference); branched apogenic peptides (U.S. Patent No. 5,591,717; incorporated herein by reference); phosphotyrosine inhibitors and non-hydrolyzable phosphotyrosine analogs (U.S. Patent No. 5,565,491; and 5,693,627; each incorporated herein by reference); agonists of RXR retinoid receptors (U.S. Patent No. 5,399,586; incorporated herein by reference); and even antioxidants (U.S. Patent No. 5,571,523; incorporated herein by reference) may also be used. Tyrosine kinase inhibitors, such as genistein, may also be linked to ligands that target a cell surface receptor (U.S. Patent No. 5,587,459; incorporated herein by reference).

VI. Pharmaceutical Compositions and Kits

Pharmaceutical compositions of the present invention will generally comprise an effective amount of a thiomolybdate compound with increased stability, particularly an alkylammonium thiomolybdate compound, such as tetrapropylammonium tetrathiomolybdate (TP-TM), dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

A. Parenteral Formulations

The agents of the present invention, such as TP-TM, will often be formulated for parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular, sub-cutaneous or other such routes, including direct instillation into a tumor or disease site. The preparation of an aqueous composition that contains one or more of such agents as an active ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Compositions comprising the agents of the present invention, such as TP-TM, can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium,

potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. Formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

Suitable pharmaceutical compositions in accordance with the invention will generally include an amount of one or more thiomolybdate compounds with increased stability, particularly an alkylammonium thiomolybdate compound, such as TP-TM, admixed with an acceptable pharmaceutical diluent or excipient, such as a sterile aqueous solution, to give a

range of final concentrations, depending on the intended use. The techniques of preparation are generally well known in the art as exemplified by Remington's Pharmaceutical Sciences, 16th Ed. Mack Publishing Company, 1980, incorporated herein by reference. It should be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less that 0.5 ng/mg protein. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

The therapeutically effective doses are readily determinable using an animal model, as shown in the studies detailed herein. Experimental animals bearing solid tumors are frequently used to optimize appropriate therapeutic doses prior to translating to a clinical environment. Such models are known to be very reliable in predicting effective anti-cancer strategies. For example, mice bearing solid tumors, such as used in the working examples herein, are widely used in pre-clinical testing. The inventors have used such art-accepted mouse models to determine working ranges of agents such as tetrathiomolybdate and TP-TM that give beneficial anti-tumor effects with minimal toxicity.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms are also contemplated, *e.g.*, tablets or other solids for oral administration, time release capsules, liposomal forms and the like. Other pharmaceutical formulations may also be used, dependent on the condition to be treated. For example, topical formulations may be appropriate for treating pathological conditions such as dermatitis and psoriasis; and ophthalmic formulations may be appropriate for conditions such as diabetic retinopathy. Of course, methods for the determination of optimal dosages for conditions such as these would be evident to those of skill in the art in light of the dosage optimization methodology disclosed in the instant specification, and the knowledge of the skilled artisan.

As described in detail herein, it is contemplated that certain benefits will result from the manipulation of the agents of the present invention, such as TP-TM, to provide them with an even longer *in vivo* half-life. Slow release formulations are generally designed to give a constant drug level over an extended period. Increasing the half-life of a drug is intended to result in high plasma levels upon administration, which levels are maintained for a longer

time, but which levels generally decay depending on the pharmacokinetics of the construct. Although currently not preferred, slow release formulations of the instant compositions and combinations thereof are by no means excluded from use in the present invention.

B. Ophthalmic Formulations

Many diseases with an angiogenic component are associated with the eye. For example, diseases associated with corneal neovascularization that can be treated according to the present invention include, but are not limited to, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma and retrolental fibroplasia, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phylectenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, mariginal keratolysis, trauma, rheumatoid arthritis, systemic lupus, polyarteritis, Wegeners sarcoidosis, Scleritis, Steven's Johnson disease, periphigoid radial keratotomy, and corneal graph rejection.

Diseases associated with retinal/choroidal neovascularization that can be treated according to the present invention include, but are not limited to, diabetic retinopathy, macular degeneration, sickle cell anemia, sarcoid, syphilis, pseudoxanthoma elasticum, Pagets disease, vein occlusion, artery occlusion, carotid obstructive disease, chronic uveitis/vitritis, mycobacterial infections, Lyme's disease, systemic lupus erythematosis, retinopathy of prematurity, Eales disease, Bechets disease, infections causing a retinitis or choroiditis, presumed ocular histoplasmosis, Bests disease, myopia, optic pits, Stargarts disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, trauma and post-laser complications.

Other diseases that can be treated according to the present invention include, but are not limited to, diseases associated with rubeosis (neovascularization of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy, whether or not associated with diabetes.

The thiomolybdate compounds of the present invention, such as TP-TM, may thus be advantageously employed in the preparation of pharmaceutical compositions suitable for use as ophthalmic solutions, including those for intravitreal and/or intracameral administration. For the treatment of any of the foregoing or other disorders, the compounds of the invention would be administered to the eye or eyes of the subject in need of treatment in the form of an ophthalmic preparation prepared in accordance with conventional pharmaceutical practice, see for example "Remington's Pharmaceutical Sciences" 15th Edition, pages 1488 to 1501 (Mack Publishing Co., Easton, PA).

The ophthalmic preparation will generally contain a thiomolybdate compound of the invention in a concentration from about 0.01 to about 1% by weight, preferably from about 0.05 to about 0.5% in a pharmaceutically acceptable solution, suspension or ointment. Some variation in concentration will necessarily occur, depending on the particular compound employed, the condition of the subject to be treated and the like, and the person responsible for treatment will determine the most suitable concentration for the individual subject. The ophthalmic preparation will preferably be in the form of a sterile aqueous solution containing, if desired, additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents and the like.

Suitable preservatives for use in such a solution include benzalkonium chloride, benzethonium chloride, chlorobutanol, thimerosal and the like. Suitable buffers include boric acid, sodium and potassium bicarbonate, sodium and potassium borates, sodium and potassium carbonate, sodium acetate, sodium biphosphate and the like, in amounts sufficient to maintain the pH at between about pH 6 and pH 8, and preferably, between about pH 7 and pH 7.5. Suitable tonicity agents are dextran 40, dextran 70, dextrose, glycerin, potassium chloride, propylene glycol, sodium chloride, and the like, such that the sodium chloride equivalent of the ophthalmic solution is in the range 0.9 plus or minus 0.2%.

Suitable antioxidants and stabilizers include sodium bisulfite, sodium metabisulfite, sodium thiosulfite, thiourea and the like. Suitable wetting and clarifying agents include polysorbate 80, polysorbate 20, poloxamer 282 and tyloxapol. Suitable viscosity-increasing agents include dextran 40, dextran 70, gelatin, glycerin, hydroxyethylcellulose,

hydroxmethylpropylcellulose, lanolin, methylcellulose, petrolatum, polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone, carboxymethylcellulose and the like. The ophthalmic preparation will be administered topically to the eye of the subject in need of treatment by conventional methods, for example in the form of drops or by bathing the eye in the ophthalmic solution.

C. Topical Formulations

In the broadest sense, formulations for topical administration include those for delivery via the mouth (buccal) and through the skin. "Topical delivery systems" also include transdermal patches containing the ingredient to be administered. Delivery through the skin can further be achieved by iontophoresis or electrotransport, if desired.

Formulations suitable for topical administration in the mouth include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

Formulations suitable for topical administration to the skin include ointments, creams, gels and pastes comprising the ingredient to be administered in a pharmaceutical acceptable carrier. Formulation for topical use, such as creams, ointments and gels, includes the preparation of oleaginous or water-soluble ointment bases, as is well known to those in the art. For example, these compositions may include vegetable oils, animal fats, and more preferably, semisolid hydrocarbons obtained from petroleum. Particular components used may include white ointment, yellow ointment, cetyl esters wax, oleic acid, olive oil, paraffin, petrolatum, white petrolatum, spermaceti, starch glycerite, white wax, yellow wax, lanolin, anhydrous lanolin and glyceryl monostearate. Various water-soluble ointment bases may also be used, including glycol ethers and derivatives, polyethylene glycols, polyoxyl 40 stearate and polysorbates.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate. Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or

spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

D. Nasal Formulations

Local delivery via the nasal and respiratory routes is contemplated for treating various conditions. These delivery routes are also suitable for delivering agents into the systemic circulation. Formulations of active ingredients in carriers suitable for nasal administration are therefore also included within the invention, for example, nasal solutions, sprays, aerosols and inhalants. Where the carrier is a solid, the formulations include a coarse powder having a particle size, for example, in the range of 20 to 500 microns, which is administered, *e.g.*, by rapid inhalation through the nasal passage from a container of the powder held close up to the nose.

Suitable formulations wherein the carrier is a liquid are useful in nasal administration. Nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays and are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, the aqueous nasal solutions usually are isotonic and slightly buffered to maintain a pH of 5.5 to 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, and appropriate drug stabilizers, if required, may be included in the formulation. Various commercial nasal preparations are known and include, for example, antibiotics and antihistamines and are used for asthma prophylaxis.

Inhalations and inhalants are pharmaceutical preparations designed for delivering a drug or compound into the respiratory tree of a patient. A vapor or mist is administered and reaches the affected area. This route can also be employed to deliver agents into the systemic circulation. Inhalations may be administered by the nasal or oral respiratory routes. The administration of inhalation solutions is only effective if the droplets are sufficiently fine and uniform in size so that the mist reaches the bronchioles.

Another group of products, also known as inhalations, and sometimes called insufflations, comprises finely powdered or liquid drugs that are carried into the respiratory passages by the use of special delivery systems, such as pharmaceutical aerosols, that hold a

solution or suspension of the drug in a liquefied gas propellant. When released through a suitable valve and oral adapter, a metered does of the inhalation is propelled into the respiratory tract of the patient. Particle size is of major importance in the administration of this type of preparation. It has been reported that the optimum particle size for penetration into the pulmonary cavity is of the order of 0.5 to 7 μm. Fine mists are produced by pressurized aerosols and hence their use in considered advantageous.

E. Kits

The present invention also provides therapeutic kits, and combined therapeutic and diagnostic kits, comprising a thiomolybdate compound with increased stability, particularly an alkylammonium thiomolybdate compound, such as TP-TM. Such kits will generally contain, in suitable container means, a pharmaceutically acceptable formulation of at least one such compound in accordance with the invention. The kits may also contain other pharmaceutically acceptable formulations, such as any one or more of a range of chemotherapeutic drugs.

The kits may have a single container means that contains the thiomolybdate compound with increased stability, such as TP-TM, with or without any additional components, or they may have distinct container means for each desired agent. Certain preferred kits of the present invention include thiomolybdate compounds with increased stability, such as TP-TM, packaged in a kit for use in combination with the co-administration of a second anti-cancer agent, such as a chemotherapeutic agent, a radiotherapeutic agent, a distinct copper chelating agent, an anti-angiogenic agent or an apoptosis-inducing agent. In such kits, the components may be pre-complexed, either in a molar equivalent combination, or with one component in excess of the other; or each of the components of the kit may be maintained separately within distinct containers prior to administration to a patient.

When the components of the kit are provided in one or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in

another container means. One of the components of the kit may be provided in capsules for oral administration.

The container means of the kit will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the more stable thiomolybdate compounds of the invention, such as TP-TM, and any other desired agent, may be placed and, preferably, suitably aliquoted. Where additional components are included, the kit will also generally contain a second vial or other container into which these are placed, enabling the administration of separated designed doses. The kits may also comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluent.

The kits may also contain a means by which to administer the thiomolybdate compounds of the invention to an animal or patient, *e.g.*, one or more needles or syringes, or even an eye dropper, pipette, or other such like apparatus, from which the formulation may be injected into the animal or applied to a diseased area of the body. The kits of the present invention will also typically include a means for containing the vials, or such like, and other component, in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials and other apparatus are placed and retained.

Further kits may comprise one or more components of a means or assay system for determining serum ceruloplasmin levels and/or instructions therefor, preferably all required assay system components and instructions for carrying out the assay. In the means or assay system for determining serum ceruloplasmin levels, preferably human serum ceruloplasmin levels, components, means and assay systems for use in the oxidase method may be used. For example, as reported in Brewer *et al.*, 1987b, Brewer *et al.*, 1987c, Brewer *et al.*, 1989 and Sunderman and Nomoto, 1970, each specifically incorporated herein by reference, for use in Wilson's Disease.

VII. Cancer and Treatment

The compositions and methods provided by this invention are broadly applicable to the treatment of any malignant tumor having a vascular component. Typical vascularized tumors are the solid tumors, particularly carcinomas and sarcomas, which require a vascular component for the provision of oxygen and nutrients. Hematologic malignancies also appear

to require angiogenesis for progression, and thus are also potentially amenable to treatment with the instant copper lowering agents. Exemplary solid tumors that may be treated using the invention include, but are not limited to, primary carcinomas of the lung, breast, ovary, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, prostate, thyroid, squamous cell carcinomas, adenocarcinomas, small cell carcinomas, melanomas, gliomas, neuroblastomas, sarcomas, such as angiosarcomas and chondrosarcomas, and the like. Metastatic tumors may also be treated using the methods and compositions of the present invention.

The present invention is contemplated for use in the treatment of any patient that presents with a solid tumor. However, in that the present invention is particularly successful in the treatment of solid tumors of moderate or large sizes, patients in these categories are likely to receive more significant benefits from treatments in accordance with the methods and compositions provided herein. In general, the invention can be used to treat tumors of about 0.3-0.5 cm and upwards, although tumors up to and including the largest tumors found in humans may also be treated.

In certain aspects of the present invention, the more stable thiomolybdate compounds of the invention, such as TP-TM, are intended as a preventative or prophylactic treatment and as a maintenance agent. There are many reasons underlying this aspect of the breadth of the invention. For example, a patient presenting with a primary tumor of moderate size or above may also have various other metastatic tumors that are considered to be small-sized or even in the earlier stages of metastatic tumor seeding. Given that TP-TM and combinations of the invention are generally administered orally or into the systemic circulation of a patient, they will naturally have effects on the secondary, smaller and metastatic tumors, although this may not be the primary intent of the treatment. Furthermore, even in situations where the tumor mass as a whole is a single small tumor, certain beneficial anti-tumor effects will result from the use of the present treatments.

The guidance provided herein regarding the most suitable patients for use in connection with the present invention is intended as teaching that certain patient's profiles may assist with the selection of patients that may be treated by the present invention, or that may, perhaps, be better treated using other anti-cancer treatment strategies. Nonetheless, the

fact that a preferred or otherwise more effective treatment is perceived to exist in connection with a certain category of patients, does not in any way negate the basic utility of the present invention in connection with the treatments of all patients having a vascularized tumor. A further consideration is the fact that the initial assault on a tumor, as provided by the therapy of the present invention, may be small in any measurable and immediate effects, but may sensitize or potentiate the tumor to further therapeutic treatments such that the subsequent treatment results in an overall synergistic effect or even leads to total remission or cure.

It is not believed that any particular type of tumor should be excluded from treatments using the present invention. It will be understood that the present methodology is widely or entirely applicable to the treatment of all solid tumors, irrespective of the particular phenotype or genotype of the tumor cells themselves. However, the type of tumor cells may be relevant to the use of the invention in combination with secondary therapeutic agents.

Those of ordinary skill in the art will understand that certain types of tumors may be more amenable to the induction of tumor stasis, regression, and even necrosis using the present invention. The phenomena is observed in experimental animals, and may occur in human treatments. Such considerations will be taken into account in conducting both the pre-clinical studies in experimental animals and in optimizing the doses for use in treating any particular patient or group of patients.

As detailed herein, there are realistic objectives that may be used as a guideline in connection with pre-clinical testing before proceeding to clinical treatment. However, this is more a matter of cost-effectiveness than overall usefulness, and is a mechanism for selecting the most advantageous compounds and doses. In regard to their basic utility, any construct or combination thereof that results in any consistent anti-tumor effects will still define a useful invention. It will also be understood that even in such circumstances where the anti-tumor effects of the instant compositions and combinations thereof are towards the low end of the range, it may be that this therapy is still equally or even more effective than all other known therapies in the context of the particular tumor targets. It is unfortunately evident to a clinician that certain tumors cannot be effectively treated in the intermediate or long term, but that does not negate the usefulness of the present therapy, particularly where it is about as effective as the other strategies generally proposed, or it may be effective after all other

conventional strategies have failed. It is not predicted that resistance to this therapy can develop.

In designing appropriate doses of the more stable thiomolybdate compounds of the invention, such as TP-TM, and combinations therewith, one may readily extrapolate from the animal studies described herein in order to arrive at appropriate doses for clinical administration. To achieve this conversion, one would account for the mass of the agents administered per unit mass of the experimental animal, and yet account for the differences in the body surface area between the experimental animal and the human patient. All such calculations are well known and routine to those of ordinary skill in the art. Accordingly, using the information provided herein, the inventors contemplate that useful daily doses of the more stable thiomolybdate compounds of the invention, such as TP-TM, for use in human administration would be between about 20 milligrams and about 200 milligrams per patient per day. Notwithstanding this stated range, it will be understood that, given the parameters and detailed guidance presented above, further variations in the active or optimal ranges would still be encompassed within the present invention.

The daily doses contemplated will therefore generally be between about 20 mg and about 180 milligrams; between about 130 mg and about 200 milligrams; between 25 and about 160 milligrams; between 50 and about 150 milligrams; between about 150 mg and about 180 milligrams; between about 30 and about 125 milligrams; between about 40 and about 100 milligrams; between about 35 and about 80 milligrams; between about 140 mg and about 190 milligrams; between about 20 and about 65 milligrams; between about 125 mg and about 195 milligrams; between about 30 and about 50 milligrams; between about 150 mg and about 200 milligrams; or in any particular range using any of the foregoing recited exemplary doses or any value intermediate between the particular stated ranges.

Although doses in and around about 60-120 mg are currently preferred in certain embodiments of the present invention, and doses in and around about 125-200 mg are currently preferred in other embodiments of the present invention, it will be understood that lower doses may be more appropriate in combination with other agents, or under conditions of maintenance, and that high doses can still be tolerated, particularly given the fact that the more stable thiomolybdate compounds of the invention, such as TP-TM, are not themselves

cytotoxic and even if certain adverse side effects do occur, this should not necessarily result in toxicity that cannot be counteracted by normal homeostatic mechanisms, which is believed to lessen the chances of significant toxicity to healthy tissues.

In certain preferred embodiments of the present invention, daily loading dosages of between about 130 mg or about 150 mg or so to about 180 mg or about 200 mg or so are administered to patients for about 2 weeks, followed by daily maintenance dosages of between about 30 mg or about 40 mg or so and about 60 mg or about 70 mg or so, or any values intermediate between the particular stated ranges. Thus, in particular aspects of the invention, loading dosages of greater than about 125 mg, greater than about 130 mg, greater than about 140 mg, greater than about 150 mg, greater than about 155 mg, greater than about 160 mg, greater than about 170 mg, greater than about 175 mg, greater than about 180 mg, greater than about 190 mg, or greater than about 200 mg or so up to the maximum dosages described herein are contemplated by the inventors as exemplary daily loading dosages for about 1 week, about 2 weeks, about 3 weeks or about 4 weeks or so, followed by daily maintenance dosages of about 20 mg, about 25 mg, about 35 mg, about 40 mg, about 50 mg, about 55 mg, about 65 mg, about 75 mg, about 80 mg or about 90 mg or so.

The intention of the therapeutic regimens of the present invention is generally to produce the maximum anti-tumor effects whilst still keeping the dose below the levels associated with unacceptable toxicity. In addition to varying the dose itself, the administration regimen can also be adapted to optimize the treatment strategy. A currently preferred treatment strategy is to administer between about 20 milligrams and about 200 milligrams of the more stable thiomolybdate compounds of the invention, such as TP-TM, or combination thereof, about 3, about 4, about 5 to about 6 or more times a day, approximately half of the time with meals, and approximately half of the time between meals. In administering the particular doses themselves, one would preferably provide a pharmaceutically acceptable composition to the patient systemically. Oral administration is generally preferred.

VIII. Other Diseases Characterized by Aberrant Angiogenesis

In addition to the prevention or treatment of cancer and solid tumors, the thiomolybdate compositions disclosed herein can also be used in preventing or treating other

diseases associated with aberrant vascularization, including, but not limited to, arthritis, diabetes, arteriosclerosis, arteriovenous malformations, corneal graft neovascularization, delayed wound healing, diabetic retinopathy, age related macular degeneration, granulations, burns, hemophilic joints, rheumatoid arthritis, hypertrophic scars, neovascular glaucoma, nonunion fractures, Osier-Weber Syndrome, psoriasis, pyogenic granuloma, retrolental fibroplasia, pterygium, scleroderma, trachoma, vascular adhesions, ocular neovascularization, parasitic diseases, hypertrophy following surgery, and inhibition of hair growth.

Each of the foregoing diseases and disorders, along with the various types of corneal neovascularization, rheumatoid arthritis and all types of tumors, as described herein, can be effectively treated by the present invention in accordance with the knowledge in the art, as disclosed in, *e.g.*, U.S. Patent No. 5,712,291 (specifically incorporated herein by reference). U.S. Patent No. 5,712,291 is specifically incorporated herein by reference to show that evidence of anti-angiogenic activity in one model, system or disease is sufficiently predictive to support the treatment of an extremely wide range of angiogenic diseases.

As disclosed in U.S. Patent No. 5,712,291, incorporated herein by reference, the compositions, methods and uses of the present invention are also intended for the treatment of animals and patients that have, or are at risk for developing, abnormal proliferation of fibrovascular tissue, acne rosacea, acquired immune deficiency syndrome, artery occlusion, atopic keratitis, bacterial ulcers, Bechets disease, blood borne tumors, carotid obstructive disease, chemical burns, choroidal neovascularization, chronic inflammation, chronic retinal detachment, chronic uveitis, chronic vitritis, contact lens overwear, corneal graft rejection, corneal neovascularization, corneal graft neovascularization, Crohn's disease, Eales disease, epidemic keratoconjunctivitis, fungal ulcers, Herpes simplex infections, Herpes zoster infections, hyperviscosity syndromes, Kaposi's sarcoma, leukemia, lipid degeneration, Lyme's disease, marginal keratolysis, Mooren ulcer, Mycobacteria infections other than leprosy, myopia, ocular neovascular disease, optic pits, Osler-Weber syndrome (Osler-Weber-Rendu, osteoarthritis, Pagets disease, pars planitis, pemphigoid, phylectenulosis, polyarteritis, post-laser complications, protozoan infections, pseudoxanthoma elasticum, pterygium keratitis sicca, radial keratotomy, retinal neovascularization, retinopathy of prematurity, retrolental fibroplasias, sarcoid, scleritis, sickle cell anemia, Sogrens syndrome, solid tumors, Stargarts disease, Steven's Johnson disease, superior limbic keratitis, syphilis, systemic lupus, Terrien's

marginal degeneration, toxoplasmosis, trauma, tumors of Ewing sarcoma, tumors of neuroblastoma, tumors of osteosarcoma, tumors of retinoblastoma, tumors of rhabdomyosarcoma, ulceritive colitis, vein occlusion, Vitamin A deficiency and Wegeners sarcoidosis.

Macular degeneration is the common name for the age-related disease where macular retinal pigment epithelium cells function less well than normal. As a result, waste removal and nutrition of the cones suffers, causing central vision loss. Macular degeneration can be further classified into two varieties: a "dry type" and a "wet type". Dry type macular degeneration occurs when the outer segments of the light sensing cones, which are continuously being shed, are unable to be digested by the pigment epithelium layer of the macula. Consequently the pigment epithelium layer swells and eventually dies after accumulating too much undigested material from the cones. Yellowish deposits of this waste material gradually develop under the retina between the choroid and pigment epithelium. In this "dry type" macular degeneration, the vision loss is characterized by gradual blurring or partial obscuration of central vision as a result of parts of the macula having begun to die, creating areas where the cones are no longer functional. Clinically, the person suffering from this type of the disease may experience relatively mild central visual distortion with straight lines appearing bent or wavy.

In the second or "wet" type of this disorder, more severe and sudden vision loss may occur. This occurs when abnormal new blood vessels or "neovascular membranes" grow from the choroid through the damaged pigment epithelium and under the macula. These neovascular membranes are fragile and are prone to hemorrhage, which results in severe distortion of the macular tissue. As a result, the light sensing cells (cones) become separated from their source of nutrients and suffer further damage due to scarring as the hemorrhage occurs over time. With this type of disorder, dark or "missing" spots in the central vision may occur rapidly and with little warning due to these hemorrhagic changes. Fortunately, intervention with laser therapy early in this process may prevent additional vision loss.

Age-related macular degeneration (AMD) is the leading cause of visual loss among adults aged 65 years or older in Western countries. Although neovascular AMD accounts for only 10% of all cases, it is responsible for 80% to 90% of legal blindness due to this disease

and is the most common cause of choroidal neovascularization (CNV) in this age population. The pathological changes leading to CNV involve the complex of tissues in the choriocapilaris, Bruch's membrane, and the retinal pigment epithelium (RPE) with secondary involvement of the neurosensory retina. Essentially anything that alters the retinal pigment epithelium and Bruch's membrane can cause CNV.

A variety of conditions other than AMD have been associated with CNV, including ocular histoplasmosis syndrome (POHS), pathologic myopia, angioid streaks, and idiopathic causes. Most histopathological studies have been performed in eyes with AMD. The histopathological feature common to many eyes that develop CNV is a break in Bruch's membrane. The capillary-like neovascularization originates from choroidal vessels and extends through these breaks. Age-related macular degeneration accounts for the largest group of patients with CNV. Most symptomatic CNV's are subfoveal and demonstrate an extremely poor natural history. Subfoveal neovascularization is defined as lesions lying under the geometric center of the foveal avascular zone (FAZ). Of untreated eyes followed for 2 years in a Macular Photocoagulation Study (MPS), only 5% had a final visual acuity better than 20/100, whereas 88% had a final visual acuity of 20/200 or worse.

Laser photocoagulation has been the mainstay of therapy for choroidal neovascularization. Through a series of well-executed randomized, prospective clinical trials, the MPS established the superiority of photocoagulation over observation for CNV in a variety of settings. Specifically, photocoagulation treatment of extrafoveal and juxtafoveal neovascular membranes in AMD and other disorders was found to be beneficial compared to the no treatment group. However, in order to treat the entire area of CNV, the ophthalmologist has to be able to identify the boundaries of the choroidal neovascular membrane. Therefore, treatment is indicated only when the boundaries of the CNV are well demarcated. Unfortunately, occult or ill-defined new vessels are the most common pattern at presentation for exudative macular lesions in AMD. In one study, visible or classic neovascular membranes involved only 23% of eyes referred for treatment. The MPS recently reported results of photocoagulation for subfoveal neovascular lesions in AMD showed benefit of laser treatment, but the difference between the treatment and observation groups was small and was seen only after two and five years. Also, as the laser energy destroys both the retina and subretinal membrane, there was a precipitous drop in visual acuity associated

with treatment. These results underline both the poor natural history of the condition and the limitations of photocoagulation as a treatment modality.

Other diseases associated with corneal neovascularization include, but are not limited to, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phylectenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, mariginal keratolysis, rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegeners sarcoidosis, Scleritis, Steven's Johnson disease, periphigoid radial keratotomy, and corneal graph rejection.

Diseases associated with retinal/choroidal neovascularization include, but are not limited to, diabetic retinopathy, macular degeneration, sickle cell anemia, sarcoid, syphilis, pseudoxanthoma elasticum, Pagets disease, vein occlusion, artery occlusion, carotid obstructive disease, chronic uveitis/vitritis, mycobacterial infections, Lyme's disease, systemic lupus erythematosis, retinopathy of prematurity, Eales disease, Bechets disease, infections causing a retinitis or choroiditis, presumed ocular histoplasmosis, Bests disease, myopia, optic pits, Stargarts disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, trauma and post-laser complications. Other diseases include, but are not limited to, diseases associated with rubeosis (neovascularlation of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy.

Another disease in which angiogenesis is believed to be involved is rheumatoid arthritis. Rheumatoid arthritis is characterized by diffuse and nodular mononuclear cell infiltration and massive hyperplasia of the stromal connective tissues, comprised of fibroblast-like cells and new blood vessels. The blood vessels in the synovial lining of the joints undergo angiogenesis. In addition to forming new vascular networks, the endothelial cells release factors and reactive oxygen species that lead to pannus growth and cartilage destruction. The factors involved in angiogenesis may actively contribute to, and help maintain, the chronically inflamed state of rheumatoid arthritis.

Factors associated with angiogenesis may also have a role in osteoarthritis. The activation of the chondrocytes by angiogenic-related factors contributes to the destruction of the joint. At a later stage, the angiogenic factors would promote new bone formation. Chronic inflammation may also involve pathological angiogenesis. Such disease states as ulcerative colitis and Crohn's disease show histological changes with the ingrowth of new blood vessels into the inflamed tissues. Bartonellosis, a bacterial infection found in South America, can result in a chronic stage that is characterized by proliferation of vascular endothelial cells. Another pathological role associated with angiogenesis is found in atherosclerosis. The plaques formed within the lumen of blood vessels have been shown to have angiogenic stimulatory activity.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Effect of TM on Cellular Toxicity

Normal and neoplastic cells in culture derive their nutrients from transport and utilization of molecules from the media to the interior of the cell. This process is not dependent on blood vessel growth, and therefore TM, TP-TM and related compounds should have no effect on cell growth rates and cell viability over a wide range of concentration, until a level that is toxic to most cells is reached. One mechanism of toxicity is the depletion of free copper levels below those required for basic cell function. For concentrations of TM, TP-TM and related compounds beyond this toxic level, both normal and neoplastic cells will be unable to survive, due to cellular toxicity.

This was confirmed in a cytotoxicity assay of MML cells (prostate cancer) and breast cancer cells. After plating cells in equal numbers in media containing various concentrations of TM ranging from 0.001 μM to 1 mM, no toxicity was observed in the range of concentrations used *in vivo*. The fraction of viable cells decreased precipitously from 100% to 15% when the concentration of TM increased from 1 μg/ml to 10 μg/ml. TM is therefore not contemplated for use a direct cytotoxic agent, as it is clear that, at the very high doses required for a cytotoxic effect, both tumor and normal cell death would occur. The serum concentrations of TM, TP-TM and related compounds needed to achieve an effective decoppering level are 100-500-fold lower than the threshold lethal level for cells. When used at efficacious decoppering doses in mice and humans with Wilson's disease, TM has not resulted in any clinically apparent direct cell toxicity.

EXAMPLE 2

Use of TM in Murine Pre-Clinical Anti-Cancer Studies

The inventors reasoned that a greater degree of copper deficiency than previously achieved is necessary to significantly inhibit angiogenesis and arrest tumor growth. This means that, in addition to decreased tumor mass, prolonged survival or tumor regression would also be observed. The studies described below, which used anti-copper approaches for tumor growth inhibition in rodents, did not fully incorporate guidelines derived from human and animal trace element studies in general, and copper studies in particular (Dick and Bull, 1945; Miller and Engel, 1960; Macilese Ammerman *et al.*, 1969; Mills *et al.*, 1958; Cox *et al.*, 1960; Dick *et al.*, 1975; Mason, 1990; McQuaid and Mason, 1991; Mills *et al.*, 1981a; Mills *et al.*, 1981b; Bremner *et al.*, 1982; Gooneratne *et al.*, 1981a, b; Jacob *et al.*, 1981).

In contrast, the present invention uses TM, TP-TM and related compounds, potent anti-copper agents. The inventors' extensive experience using zinc as a treatment in sickle cell anemia (where the inventors described the first human cases of zinc induced copper deficiency; Brewer *et al.*, 1983), zinc as an anti-copper treatment in Wilson's disease (Brewer *et al.*, 1989; Brewer, 1995a), and TM for the initial anti-copper treatment in Wilson's disease (Brewer *et al.*, 1983; Brewer, 1992; Brewer *et al.*, 1994b; Brewer *et al.*, 1995b) was employed in the development of algorithms to achieve effective copper deficiency. As the

developers of TM for clinical use, the inventors have acquired significant experience with TM in both animals and humans.

A. Injection of Tumor Cells into C567Bl/C6 Mice

Under these guidelines, anti-angiogenesis therapeutic studies were conducted using TM in a mouse tumor model. This study involved subcutaneous or intramuscular injection of tumor cells into young adult C57B1/6J mice. Two tumors were used, a mouse sarcoma, MCA205, and a mouse melanoma, B16Bl6. The MCA 205 tumor cell suspension was injected subcutaneously into C57Bl6 mice. After the tumors were palpable, copper deficiency was induced by use of TM in half the animals, using Cp levels to monitor copper status. The other half were sham treated. Tumor growth was compared between the untreated control group and mice treated with sufficient TM to reduce Cp to about 10% of normal controls.

A significant effect on reducing the growth rate of tumors, and on reducing final tumor size and weight, was obtained with TM. Tumor growth was slowed by the treatment, although not halted, in the relatively brief period of observation (16 days). The tumors in TM-treated mice weighed significantly less than the tumors in control mice, normalized to the total weight of the animal. The results were similar in both tumor types. Thus TM reduced tumor growth and mass, presumably *via* its anti-angiogenesis mechanism, as there are no cytotoxic effect at the doses employed.

However, this experimental design can be improved further to evaluate the potential efficacy of TM as an anti-tumor agent for the following reasons: 1) In the mouse, tumors over 1 cm can become a life-threatening burden, and often ulcerate. However, in both mice and humans, it is tumors of this size and larger which are most dependent upon angiogenesis for sustained growth; and 2) The period between detecting the tumor at 2-3 mm and at 1.5 cm is relatively brief in the mouse, consisting of only a few days. As it takes time to deplete the tumor mass of copper (especially since most tumors sequester high levels of copper), the tumor may support significant angiogenesis from its own stores, prior to the achievement of near complete copper depletion.

B. HER2-neu Transgenic Mice

Therefore, an animal protocol was designed to test the effectiveness of TM in retarding or preventing clinically evident tumors in cancer prone female HER2-neu transgenic mice (Guy *et al.*, 1992; Muller *et al.*, 1988). These mice are normal at birth and in infancy, but because of the transgene, one hundred percent of these mice develop mammary tumors between 4-8 months of age (median 205 days) (Guy *et al.*, 1992). A major additional reason for choosing this model is that the natural history of these murine mammary tumors is remarkably akin to the clinical behavior of untreated breast cancer in humans. The *HER2-neu* mouse tumors develop after a long latency (now known to be likely due to mutagenesis of the transgene and not to over expression) and they remain primarily local-regional until they achieve a large size (often > 2.5 cm) before metastasizing mostly to lung. The time of onset of tumors and the quality and quantity of tumor vessels between the treatment and control groups were compared.

After breeding 3 founder transgenic females with 3 transgenic males, the female progeny was segregated into 2 groups: one group had 15 treatment and the other 22 control animals. The percentage of disease-free mice in the untreated control group was compared to a group of the same transgenic mice treated with 0.5-1.0 mg TM by gavage daily, starting at 100 days of age. Treatment with TM was initiated approximately 80-100 days before the tumors would have become clinically evident, so that the treated animals are rendered copper deficient throughout the key period of tumor development when angiogenesis may begin to be required. This prevents tumors from sequestering large amounts of copper from which they could sustain angiogenesis, even in the face of falling total body copper.

With a median follow-up of 260 days, none of the TM-treated mice developed clinically overt tumors, whereas 70% of the genetically identical controls had shown tumors in the same follow-up period. Fifty percent of control mice had developed clinically apparent tumors by age 218 days ($p < 0.02$). Whereas the controls started exhibiting tumors beginning at age 153 days, the TM-treated animals showed no tumors until TM-therapy was discontinued and copper levels were allowed to drift upwards ($p<0.0146$).

Monitoring of blood copper levels in the treatment group by use of a surrogate indicator, ceruloplasmin (Cp) (measured by spectrophotometric activity assay), revealed that

Cp had decreased to below 40% of baseline. In this strain of mice, there was no anemia observed when the copper decreased to this level. A separate group of 4 treatment animals was given higher doses of TM between 1.0-1.5 mg over 2-4 weeks. The animals who received 1.25-1.5 mg died after 1-3 weeks of therapy. Autopsies revealed that one animal died of aspiration pneumonia (ascribed to gavage accident) whereas 2 animals died of renal tubular necrosis and pulmonary hemorrhages, with clear evidence of vascular injury to these tissues on necrospy. These studies suggest that 1.0 mg/day is the maximum tolerated dose (MTD) for TM in adult mice (average weight 32 grams), when prolonged treatment is planned.

In order to test whether mammary tumors in the control mice could be decreased in size with TM, 3 of the control animals were treated after the tumors were well established (> 1.5 cm largest dimension). In 2/3 cases, the tumors were shrunk significantly by 25% and 50%.

Next, the possibility that prolonged TM therapy had somehow damaged the breast tissue so tumors could not possibly arise in the treatment animals due to lack of a target was determined. After 80% of the control animals and none of the treatment animals had developed tumors, the control animals were released from treatment. Clinically overt mammary tumors began to develop in this cross-over group of mice (which had been previously disease-free), within 18 days, suggesting that indeed the tumor initiation event in the target tissue had taken place but expansion of the tumor to a clinically detectable mass was not possible in the copper deficient state.

Microscopic analyses of the mammary glands of the TM-treated mice revealed a number (1-8) of small "micro-tumors" approximately 3-10 cell-layers thick which failed to vascularize. However, upon release from TM therapy, these micro tumors grew very rapidly into palpable masses that were briskly vascularized. Although not all the cellular and molecular details have yet been elucidated, it is clear that in this important carcinogenesis model, copper deficiency inhibited the angiogenic switch, or a step closely downstream from it, as none of the micro-tumors of the treated mice were vascularized.

In summary, decreasing copper availability decreases and eventually arrests solid tumor growth, including growth of metastases. The decrease in copper availability needed to decrease tumor growth in humans without Wilson's disease was ascertained as described herein below. The success of TM, TP-TM and related compounds as anti-tumor agents is based, at least in part, on the relationship between the degree of copper deficiency required to obtain efficacy, and the toxicity of that degree of copper deficiency. As tumor angiogenesis is abrogated when mild copper deficiency ensues, TM, TP-TM and related compounds are remarkably effective agents for several different oncologic applications. Other settings contemplated for TM, TP-TM and related therapy include, but are not limited to, maintenance therapy following chemotherapy or bone marrow transplantation, in patients who are elderly or otherwise ineligible to receive chemotherapy, or as a cytostatic agent in high-risk individuals (inflammatory cancer, multiple positive nodes).

C. Nude Mice With Transplanted Tumors

In this study, the ability of tetrathiomolybdate (TM) to abrogate or retard the growth of tumors in the mammary pads of nude mice after orthotropic injection of human breast cancer cells was studied. The highly angiogenic inflammatory breast cancer cell line called SUM149 was selected for this study. Given its high propensity to form palpable tumors within 2 weeks of injection into the mammary of 80-100% of the nude mice injected, this cell line posed a stringent test of the ability of TM to impair tumor progression.

Three groups of 5 nude mice each were set up in separate cages. Groups 2 and 3 received TM in the drinking water to average an intake of 1.2 mg/day/mouse, starting at day -7. Groups 1, 2, and 3 were injected on day 0 with 10^6 SUM149 breast cancer cells in the second thoracic mammary fat pad. On day 34, as no palpable tumors were noticeable in any of the mice in the treatment groups, TM was skipped on groups 2 and 3, and then TM was restarted on group 2 at a dose of 0.6 mg/day/mouse, while TM was resumed at full dose (1.2 mg/mouse/day) in group 3.

The developing tumors were measured approximately twice weekly, and the average of the product of the bi-dimensional perpendicular diameters for each group versus time was determined. The tumors in the control group grew relatively rapidly and all animals developed tumors. In contrast, for the treatment groups, no tumors were palpable until TM

was skipped on day 34. Thereafter, the tumors grew more rapidly in the group that received 1/2 dose of TM, which is known to only decrease the copper levels by 50%. In contrast, only very small tumors have grown in the group treated with TM at full dose, which is defined as 1.2 mg/day/mouse. This dose decreases copper to approximately 10-20% of baseline levels. It is this degree of copper deficiency, well tolerated in both mice and humans, which appears to be required to inhibit tumor angiogenesis.

This study supports the idea that TM, TP-TM and related compounds probably inhibit angiogenesis at least in 2 ways: one is by inhibition of the "angiogenic switch", and the other is by inhibition of bulk tumor angiogenesis. Skipping TM on day 34 enabled the angiogenic switch to activate and begin to bring a vessel to the tumor cluster which had been injected in copper deficient nude mice. This activation of the switch enables some tumor growth, although it is clear that the group treated with TM has much slower tumor growth, and appears to be reaching a plateau. This study also validates oral administration of TM, TP-TM and related compounds in the drinking water of the nude mice.

EXAMPLE 3

Phase I/II Clinical Trial of TM as Anti-Cancer Therapy

A. Introduction

Patients with metastatic solid tumors often have very limited treatment options due to the cumulative toxicity of cytoreductive chemotherapy and drug resistance. Following the pre-clinical work detailed above, which showed efficacy for the anti-copper approach in mouse tumor models, a Phase I clinical trial was conducted in 18 patients with metastatic cancer who were enrolled at 3 dose levels of oral tetrathiomolybdate (TM; 90, 105, and 120 mg/day) administered in 6 divided doses with and in-between meals. Serum ceruloplasmin (Cp) was used as a surrogate marker for total-body copper. As anemia is the first clinical sign of copper deficiency, the goal of the study was to reduce Cp to 20% of baseline value, without reducing hematocrit by more than 80% of baseline. Cp is a reliable and sensitive measure of copper status, and TM was non-toxic when Cp was reduced to 15-20% of baseline. The level III dose of TM of 120 mg/day was effective in reaching the target Cp, without added toxicity. TM-induced mild copper deficiency achieved stable disease in 5 out of 6 patients who were copper deficient at the target range for at least 90 days.

1. Toxicity

The pharmacological effects of TM, TP-TM and related compounds are completely specific to copper. As they have no detectable effects on other minerals, toxicity is then directly related to copper deficiency. Other than copper deficiency, at the doses employed, there is no toxicity described in animals. In humans, there are two reports of reversible anemia in Wilson's disease patients taking TM for maintenance therapy at doses of 30-40 mg 6 times per day. In treating 45 patients with Wilson's disease for eight weeks with TM as initial therapy for Wilson's disease, five cases of reversible anemia (11.1%) were observed. The anemia is due to decreased heme synthesis as a result of copper depletion in the bone marrow. The patients whose blood showed the most severe reduction in Cu levels exhibit this anemia.

As mentioned above, the copper status of an animal or human undergoing TM, TP-TM and related therapies cannot be followed by serum copper alone, because the complex of TM, copper, and albumin, is cleared more slowly from the blood than it accumulates, until a high copper steady state is reached. This complexed copper is however not available for cellular uptake, and is gradually cleared from the body *via* the urine and the bile, without participating in any cellular copper-dependent processes, such as angiogenesis. The inventors have developed guidelines for monitoring the copper balance in humans during TM, TP-TM and related therapies, according to the recognized three stages of copper deficiency (Brewer, 1992; Brewer *et al.*, 1991a), as follows.

First Stage - Chemical Copper Deficiency

During this stage, the serum ceruloplasmin (Cp) activity is decreased up to approximately 5-10% of baseline. Cp, a copper containing protein, is synthesized in the liver and Cp synthesis is decreased during copper depletion. This stage of copper deficiency, although measurable in the laboratory, has no clinical signs or symptoms. The Cp must be below a few percent of normal before early clinical copper deficiency ensues.

Second Stage - Mild Clinical Copper Deficiency

After the Cp is held at 0-5% for a period of 5-10 days, the first clinical signs of copper deficiency may appear. These are mild neutropenia and hypochromic microcytic red cell

changes. Both the white blood count and hematocrit fall to approximately 75-85% of baseline. Copper is required for heme synthesis, so the morphological changes seen in the peripheral smear are similar to those characteristic of iron deficiency. The anisocytosis and poikilocytosis exacerbate as copper deficiency becomes more severe. The onset of this mild anemia and neutropenia is gradual and often entirely asymptomatic.

Third Stage - Moderate to Severe Clinical Copper Deficiency

Typically when Hct < 70% of baseline, more severe clinical signs and symptoms resulting from inadequate hematopoesis ensue. These are loss of appetite, weight loss, diarrhea, impaired melanogenesis with subsequent loss of hair color, rarely cardiac arrhythmias.

The inventors reasoned that mild chemical copper deficiency, (a condition which is extremely well tolerated by humans for seemingly long periods of time) has efficacy as a strategy to inhibit solid tumor angiogenesis. Modulation and careful monitoring of the degree of copper deficiency to establish surrogate end-points of efficacy of TM, TP-TM and related compounds is therefore an important element of this approach.

The inventors have very good evidence-based knowledge about the various stages of relative copper deficiency, the dividing line between chemical and clinical copper deficiency, and appropriate protocols on what to measure to assess copper status. Since the patients are closely monitored, the Phase I study has so far proven to be quite safe. The major therapeutic issue discerned was that the tumor angiogenic requirements for copper are significantly higher than essential cellular housekeeping needs for copper.

2. Pharmacodynamics

TM is administered orally with and without meals, and is well absorbed under the latter condition. TM forms a tripartite complex with copper and protein, thereby binding copper in food when administered with meals, preventing copper absorption, or in the bloodstream (TM-Cu-albumin) after absorption, preventing cellular copper uptake. In patients with normal copper metabolism, a stoichiometric 1:1 relationship between non-ceruloplasmin plasma copper (potentially available for angiogenesis) and plasma molybdenum is expected, with 6 daily doses of 10-20 mg each. The dose level of TM, TP-

TM and related compounds that will result in a steady state of mild copper deficiency varies within the range of 40-90 mg daily for most individuals. The complex of TM-Cu-protein is slowly excreted predominantly in bile, with a small amount excreted also in the urine. Twenty-four hour urine measurements of Mo and Cu will help determine the rate of elimination of the tripartite complex.

B. Methods

1. Patients

Eighteen adults with metastatic solid tumors, exhibiting measurable disease, life expectancy of 3 or more months, and at least 60% Karnofsky performance status were enrolled. Patients with effusions or bone marrow involvement as the only manifestations of disease, and those who had severe intercurrent illness requiring intensive management or were transfusion dependent, were excluded. Patients had to have recovered from previous toxicities, and had the following requirements for laboratory parameters: WBC $\geq 3{,}000/mm^3$, ANC $\geq 1{,}200/mm^3$, Hct $\geq 27\%$, Hgb ≥ 8.0 gm/dl, platelet count $\geq 80{,}000/mm^3$, bilirubin < 2.0 mg/dl, AST/ALT < 4 times the upper limit of institutional norm, serum creatinine < 1.8 mg/dl or calculated creatinine clearance ≥ 55 ml/min, calcium < 11.0, albumin ≥ 2.5 gm/dl, PT < 13 sec., and PTT < 35 sec. Other requirements were demonstrable progression of disease in the previous 3 months, after standard treatments such as surgery, chemotherapy, radiotherapy, and/or immunotherapy, or progressive disease after declining conventional treatment modalities.

2. Treatment Schema: Doses and Escalation

Three dose regimens were evaluated. All dose levels consisted of TM 20 mg given 3 times daily with meals plus an escalating (levels I, II, and III) in-between meal dosing, given 3 times daily, for a total of 6 doses per day. Loading dose levels I, II, and III provided TM at 10 mg, 15 mg, and 20 mg, 3 times daily between meals, respectively, in addition to the 3 doses of 20 mg each, given with meals, at all dose levels.

Baseline Cp was taken as the nearest Cp measurement to day 1 of treatment, including day 1, since the blood was drawn pre-TM, for all patients. The target Cp reduction was defined as 20% of baseline Cp. Due to Cp assay variability of approximately 2%, a change of Cp to 22% of baseline was considered as achieving the desired reduction of copper. In

addition, if the absolute Cp was less than 5 mg/dl, then the patient was considered as having reached the target Cp. No patient reached the target due to an absolute Cp of less that 5 mg/dl, without also being at least 78% reduced from baseline. After reaching the target copper deficient state, TM doses were individually tailored to maintain Cp within a target window of 70-90% reduction from baseline.

Six patients were to be enrolled at each dose level. After 4 patients were enrolled at level I, if one patient experienced dose-limiting toxicity (DLT) (defined as Hct < 80% of baseline), 2 more patients were enrolled at level I. If no DLT was observed, patients were enrolled at the next dose level. Treatment was allowed to continue beyond induction of target copper deficiency, if the patients experienced a partial or complete clinical response or achieved clinical stable disease by the following definitions. Complete response is defined as the disappearance of all clinical and laboratory signs and symptoms of active disease. Partial response is defined as a 50% or greater reduction in the size of measurable lesions defined by the sum of the products of the longest perpendicular diameters of the lesions, with no new lesions or lesions increasing in size. Minor response is defined as a 25-49% reduction in the sum of the products of the longest perpendicular diameters of one or more measurable lesions, no increase in size of any lesions and no new lesions; stable disease is any change in tumor measurements not represented by the criteria for response or progressive disease, which is defined as an increase of 25% or more in the sum of the products of the longest perpendicular diameters of any measurable indicator lesions, compared to the smallest previous measurement or appearance of a new lesion. Because copper deficiency is not a cytotoxic treatment modality, the patients who provide information about the efficacy of TM for long-term therapy, in this population of patients with advanced cancer, are primarily those who remained within the target Cp window of (20±10)% of baseline for over 90 days, without disease progression

3. Monitoring of Copper Status

A method was required to monitor copper status easily and reliably, so that TM dose could be adjusted appropriately during this trial. With TM administration, serum copper is not a useful measure of total-body copper, because the TM-copper-albumin complex is not rapidly cleared, and the total serum copper (including the fraction bound to the TM-protein complex) actually increases during TM therapy (Brewer *et al.*, 1991a; 1994b; 1996). The

serum ceruloplasmin level obtained weekly was used as a surrogate measure of total-body copper status. The serum Cp level is controlled by Cp synthesis by the liver, which, in turn, is determined by copper availability to the liver (Linder *et al.,* 1979). Thus, as total-body copper is reduced, the serum Cp level is proportionately reduced. The serum Cp level is in the range of 20-35 mg/dl and 30-65 mg/dl for normal controls and cancer patients, respectively. The objective as this trial was to reduce Cp to or below 20% of baseline, and to maintain this level, within a window spanned by (20±10) % of the baseline Cp, with typical Cp values in the range of 7-12 mg/dl. Since there appears to be no untoward clinical effects from this degree of copper reduction, this level of copper deficiency has been termed "chemical copper deficiency". The first indication of true clinical copper deficiency is a reduction in blood cell counts, primarily anemia, as copper is required for heme synthesis as well as cellular proliferation (Brewer *et al.*, 1996). Thus, the copper deficiency objective of this trial was to reduce the Cp to 20% of baseline or below, without decreasing the patient's hematocrit or WBC to below 80% of the baseline value at entry.

4. Toxicity, Follow-Up, and Disease Evaluation

Complete blood counts, liver and renal function tests, urinalyses, and Cp level (by the oxidase method) were performed weekly for 16 weeks, then bi-weekly. Physical examinations and evaluations of toxicity were carried out every 2 weeks for 8 weeks, then every 4 weeks for the duration of therapy. Toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria. As TM, TP-TM and related compounds are not cytotoxic drugs at the doses employed, and as TM has already been given to humans without any other toxicities than the ones described in detail above, the majority of toxicities, should they arise, are not expected to be due to TM, TP-TM and related compounds. Nevertheless, therapy will be discontinued and the patient removed from the study if grade 3 or higher toxicities of any type are observed, whatever the probable etiology. For grade 2 toxicities, an attempt will be made to establish their etiology. If routine support care measures do not alleviate the conditions, the drug will be discontinued and the patients removed from the study.

Extent of disease was evaluated at entry, at the point of achievement of copper deficiency, defined as Cp at or below 20% of baseline, and every 10-12 weeks thereafter. Computer-assisted tomography or magnetic resonance imaging were used as appropriate for

conventional measurement of disease at all known sites and for evaluation of any potential new sites of disease. Angiogenesis-sensitive ultrasound with 3–dimensional Doppler analyses was employed in select cases, as adjunct to conventional imaging, to evaluate the blood flow to the tumors at different time points.

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5. TM Preparation and Storage

TM was purchased in bulk lots suitable for human administration (Aldrich Chemical Company, Milwaukee, WI). As TM is slowly degraded when exposed to air, with oxygen replacing the sulfur in the molecule, rendering it inactive (Brewer *et al.,* 1991a; 1994b; 1996), it was stored in 100-gram lots under argon. At the time a prescription was written, the appropriate dose of TM was placed in gelatin capsules. Previously, it was shown by the inventors that TM dispensed in such capsules retained at least 90% of its potency for eight weeks (Brewer *et al.,* 1991a). Thus, TM was dispensed to each patient in eight-week installments throughout the trial.

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6. Measurement of Blood Flow

Blood flow was measured by ultrasound in select patients with accessible lesions, at the time they became copper deficient, and at variable intervals of 8-16 weeks thereafter. 3D scanning was performed on a GE Logiq 700 ultrasound system, with the 739L, 7.5 MHz linear array scanhead. The scanning and vascularity quantification techniques were as previously described (Carson *et al.,* 1998; LeCarpentier *et al.,* 1999).

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C. Results

1. Patient Characteristics

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Eighteen eligible patients, 10 males and 8 females, with 11 different types of metastatic cancer who had progressed through or (in one case) declined other treatment options were enrolled in the trial, in the order in which they were referred. Six, 5, and 7 patients were enrolled at the 90, 105, and 120 mg/day drug levels, respectively, following the protocol dose escalation schema. One patient originally assigned to the 105 mg level was removed early to pursue cytotoxic chemotherapy, due to rapid progression of disease. This same patient was later retreated at the 120 mg level for a longer duration, and thus is counted only at the 120 mg drug level for the analyses. The average age was 59; the average baseline

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Cp was 47.8 mg/dl, which is elevated with respect to normal reflecting the patients' disease status.

2. Toxicity

There were no cardiac, pulmonary, GI, renal, hepatic, hematologic, infectious, skin, mucosal, or neurologic toxicities observed for Cp levels at or above 20% of baseline. Mild (greater than 80% of baseline Hct) reversible anemia was observed in 4 patients for Cp levels between 10-20% of baseline. Two of these patients had been treated with cytotoxic chemotherapy and two patients had evidence of extensive bone marrow involvement with their disease at the time of entry into the trial. Although in the latter two of these cases, the anemia was most likely due to causes other than treatment, TM was discontinued temporarily until Hct was restored to acceptable levels with transfusion of 2 units of packed RBCs. In one patient, it is very likely that the copper deficiency caused by TM produced the anemia. Stopping the drug allowed the hematocrit to recover within 5-7 days without the need for transfusion; at the patient's request, TM was restarted at a lower dose, without further complications of anemia. Several patients experienced transient, occasional sulphur-smelling burping, within 30 minutes of TM ingestion. No additional toxicities of any type were observed with long-term maintenance of mild clinical copper deficiency over 8-15 months. Of note, no evidence of GI or other mucosal bleeding or impaired healing of minor trauma were observed with long-term therapy. One pre-menopausal patient with extensive metastatic renal cancer experienced normal menstrual periods during TM therapy, including 2.5 months of observation while copper deficient with Cp < 20% of baseline.

3. Ceruloplasmin as a Surrogate Measure of Copper Status

The response of Cp as a function of time on TM therapy, expressed as the ratio of Cp at time t to the baseline Cp level for each patient enrolled at the 90 mg/day, 105 mg/day, and 120 mg/day dose levels, was determined. The average time to 50% reduction of Cp is 30 days. Increasing the in-between meals dose from 10 mg 3× daily to 15 mg or 20 mg 3× daily had no significant effect on the rate of decrease of the Cp level, reaching a level of 50% baseline at a mean of 30 days (median = 28 days). The response of Cp to TM therapy as a function of time exhibited only minor fluctuations; when TM was discontinued, a rapid rise in Cp, within 48 hours, was observed.

Four patients were removed from study due to progression of disease prior to achieving target Cp of 20% of baseline, while the remaining 14 patients achieved the target Cp level. Since all 14 patients who achieved the target Cp level wished to remain on study, they were allowed to do so, according to the protocol, as long as they did not exhibit disease progression or toxicity. The TM doses were adjusted in these patients to maintain the Cp between 10-20% of baseline. These patients provide the preliminary evidence of efficacy and of long-term tolerance of this approach.

4. Dose Adjustments to Maintain Target Cp

In order to maintain a Cp target of 20% of baseline and to prevent absolute Cp values less than 5 mg/dl, TM doses were adjusted. Due to a routine 7-day turn-around for the Cp test, these dose changes were made approximately 7-10 days after the blood for the Cp measurement was taken. After achieving the target Cp, the in-between meals dose was typically decreased by 20 mg. Further decreases of 15-30 mg were necessary during long-term therapy. A patient with metastatic chondrosarcoma secondary to radiation treatment for breast cancer on long-term therapy has stable disease after 12 months of copper deficiency, with stable quality of life. One biopsy-proven metastatic nodule on the third digit is easily measurable and has been stable. Interestingly, this patient has required only a minor adjustment to the TM dose from the initial loading dose level to maintain the target Cp throughout this relatively long period.

The management of long-term therapy with TM, TP-TM and related compounds to maintain a Cp target of 20% of baseline can be readily achieved, as described for the following representative patients over approximately 100 days of therapy with TM. One patient has so far required only decreases in dose 60 days apart. In order to prevent the Cp from falling below 5 mg/d, this patient will likely require a decrease in TM dose in the future. Most patients have required both increase and decrease in dose, during long-term therapy. For example, the TM dose has been increased after day 100 to prevent a drifting of the Cp above the target range. Heterogeneity of diet and tumor behavior (such as tumor cell lysis) may account for the individual variability in dose adjustment needs. Other sites of suspected disease in the chest also remain stable. In conclusion, the Cp response to TM therapy evaluated weekly is not brittle or subject to wide fluctuations.

5. Measurement of Response of Metastatic Cancer to TM

Clinical Evaluation

Although the patients received different initial loading doses of TM, the Cp maintenance window of (20±10)% of baseline was used in all groups, regardless of the loading dose. Patients who maintained this degree of copper deficiency through tailored adjustments of the dose for over 90 days, are likely to reflect the anti-angiogenic activity of TM, TP-TM and related compounds against their tumors. The period of 90 days is selected for 2 main reasons. First, TM, TP-TM and related compounds are not cytotoxic to either cancer or endothelial cells and mainly impair endothelial cell function and pro-angiogenic factor production. This mechanism of action is expected to have a very slow effect on the size of tumor masses. Second, as tumors sequester copper, the microenvironment of the tumor is expected to take a longer time to be rendered copper deficient.

Fourteen patients achieved the target copper deficiency prior to disease progression or other disease complications. Of these, 8 patients either progressed within 30 days of achieving copper deficiency or have had stable disease for fewer than 90 days; it is unlikely that most of these tumors experienced an anti-angiogenic environment long enough to evaluate clinical response to this type of therapy. In all patients who were removed from protocol due to disease progression or choice, and in one patient due to the need for abdominal surgery to relieve a small bowel obstruction, much more rapid rates of progression of disease were noted clinically, after discontinuation of TM therapy.

The remaining 6 patients experienced stable disease (5/6) or progression of disease at one site, with stable disease elsewhere (1/6). Two patients who have stable disease by standard criteria also experienced complete disappearance of some lung lesions and decrease in size of other lung lesions during observation periods at target Cp of 120 and 49 days. The 5 patients on long-term (over 90 days) maintenance therapy with stable disease have been copper deficient between 120 and 413 days at the time of this analysis.

Radiologic Evaluation

Serial evaluations of tumor masses by conventional imaging with CAT scan or MRI revealed that the radiographic appearance of the certain masses changed significantly over time. In particular, areas of presumed central necrosis (corresponding to lower attenuation of

the X-ray signal) were observed in a variety of tumor types, most notably renal cell cancer, angiosarcoma, and breast cancer. Seeking to evaluate the blood flow to the tumors as a function of time during copper deficiency on long-term TM therapy, lesions accessible to ultrasound were imaged with color flow 3-dimensional ultrasound at the onset of copper deficiency, and at intervals of 2-4 months thereafter.

Conventional CAT scan images and blood-flow sensitive 3D-ultrasound were compared in a rib metastasis from a renal cell carcinoma upon reaching target copper deficiency and 8 weeks later. A CAT scan showed this lesion to be of a stable size over time, although a more distinct region of (probable) central necrosis is observed 8 weeks after reaching target copper deficiency. In comparison, a decrease in blood flow to this mass by 4.4-fold in this period of approximately 8 weeks was detected by the 3D-ultrasound. In addition to the mass studied by these two techniques, this patient had extensive disease in the chest, pelvis, and femurs.

TM in Combination With Other Treatment Modalities

During the long-term maintenance of copper deficiency, additional treatment modalities were added to TM, as deemed appropriate for the optimal management of the patients. A patient with previously untreated metastatic breast cancer is doing well with a good to excellent quality of life after 12 months of treatment. This patient had metastases in the paratracheal, posterior cervical, and retroperitoneal lymph node chains, but had declined all cytotoxic therapy. The patient had stable disease for over 6 months on TM treatment, when, due to slight increase (less than 25% of baseline) in the bidimensional size of the paratracheal and retroperitoneal nodes, was begun on concurrent trastuzumab therapy, after this drug became commercially available. This patient showed a rapid response to trastuzumab at all sites of disease: after 1 cycle, there was a clinical complete response in the neck, and after 3 cycles of trastuzumab, there was radiologic confirmation of complete response at all previous sites of disease. The patient remains on TM, but the trastuzumab was discontinued after 6 doses. The patient continues to maintain status as a complete responder on TM alone for 3 months after discontinuation of trastuzumab therapy. Because the complete response was achieved after addition of trastuzumab therapy, this patient is classified as having only stable disease on TM.

Two patients with extensive angiosarcoma of the face and scalp achieved stable disease on TM. In one patient with severe chronic bleeding from an ocular lesion which threatened the orbit, interferon-alpha 2 (IFN-α) was added to TM to attempt to enhance tumor response. Given the suggestion that, based on studies of progressing hemangiomas, the use of low-dose interferon may be efficacious for the treatment of hemangioma (Takahashi *et al.*, 1994), IFN-α was administered to both of these patients at a dose of 500,000 units subcutaneously twice a day. Radiotherapy was also given to these 2 patients while on TM, to attempt to control actively bleeding (but not progressing) lesions. Both patients had disease stabilization for over 60 days, with one of them remaining with stable disease for over 5 months, prior to discontinuation of therapy due to patient choice. No exacerbation of toxicity was observed by addition of any of these treatment modalities to TM.

This is the first human trial of induction and maintenance of copper deficiency with tetrathiomolybdate as an anti-angiogenic therapy for cancer. In a group of patients with advanced cancer, it was demonstrated that TM is remarkably non-toxic when Cp is lowered to 10-20% of baseline levels for up to 17 months of treatment. The only drug-related toxicity observed was mild anemia in one patient, which was easily reversible with adjustment in the TM dose to bring the Cp level to the desired target. In spite of the diverse roles that copper plays in diverse essential biological processes including heme synthesis, superoxide dismutase and cytochrome function, no lasting significant adverse effects were observed upon reduction of Cp to approximately 20% of baseline. This level of copper reduction constitutes the lower limit of chemical copper deficiency and the beginning of mild clinical copper deficiency, the first manifestation of which is mild anemia.

The use of serum Cp level obtained by the oxidase method, an inexpensive and widely available test, was validated as a sensitive and reliable surrogate marker of total-body copper status during TM therapy. Using the six times per day dose regimen, and initial TM doses ranging from 90 to 120 mg per day, the serum Cp was reliably lowered to 50% of baseline in 17 out of 18 patients treated and to 20% of baseline in 14 out of 18 patients. Reduction to 50% of baseline was achieved on the average in 30 days, with further reduction to Cp levels of 5-10 mg/dl taking 20-30 days. Although this rate of decrease in Cp is reasonable for the initial treatment of early malignant lesions or in the adjuvant setting, in widely metastatic advanced cancer, this rate of decrease will be accelerated to prevent some

disease progression during induction of copper deficiency in a significant number of patients. Since loading dose variations of 90 to 120 mg per day do not appear to affect the rate of Cp reduction, and given the typical daily intake of copper with food, higher doses in-between meals will be required to accelerate the rate of induction of copper deficiency.

As the Cp response to TM-induced copper deficiency is monotonic and exhibits little inter-subject variability, there is essentially no risk of sudden changes or unpredictable fluctuations that might make dose management difficult. Following Cp levels once every one to two weeks is adequate to monitor copper status. As a corollary, overtreatment is easily detectable and correctable.

As a result of this study it is apparent that, with the present TM dose regimens, there is considerable lag between initiation of TM therapy and reduction of copper levels in tumors to a likely anti-angiogenic level. Further retarding the ability to reach anti-angiogenic levels of copper deficiency is the likelihood that most tumors sequester copper (Arnold and Sasse, 1961; Apelgot *et al.*, 1986; Gullino *et al.*, 1990; Fuchs and Sacerdote de Lustig, 1989). Thus, additional time may be required to deplete the tumor micro-environment to an effectively low level of copper, defined as a level low enough to inhibit angiogenesis. Patients with very rapidly progressive large tumors may therefore benefit from additional treatment modalities, as described herein, in addition to anti-angiogenesis therapy.

Furthermore, initially effective anti-angiogenesis may cause brisk tumor necrosis, resulting in the release of additional copper from the dying cells. In the case of one patient, a transient rise in Cp was observed at approximately the same time as the ultrasound suggested that the large tumor mass might be undergoing central necrosis due to a significant decrease in blood flow. Thus, a period of 60-90 days of Cp at the target level of 20% of baseline is a reasonable starting point for evaluation of response to anti-copper therapy. In the two patients who exhibited partial regression of lung lesions, tumor control may have begun earlier. It is also interesting to note that, in both of these patients, the lung parenchymal metastases were the sites of tumor regression. It is possible that mild clinical copper deficiency impairs superoxide dismutase function (Culotta *et al.*, 1997) so that under conditions of high oxidant stress, such as those present in the lung, the metastatic foci are more susceptible to oxidative damage.

In spite of individual differences, the use of 3D-ultrasound to determine the total blood flow to a given mass demonstrates that maintenance of mild copper reduction to 20% of baseline induced for at least 8 weeks appears sufficient to alter tumor blood flow. Due to the relative insensitivity of computer-assisted tomography to the blood flow or metabolic status of the lesions, parallel imaging modalities, as demonstrated here for 3-D ultrasound, are preferred to assess functional response in addition to tumor size.

These studies show that that the size of solid tumors of a variety of types may be stabilized or decreased by TM, given sufficient time in a state of mild clinical copper deficiency represented by a decrease in Cp to or below 20% of baseline, as defined by this study. Among the patients who were maintained at the target Cp level for more than 90 days, a significant proportion of cases (5/6) were stabilized, with no detriment to their quality of life. In this population of patients with advanced disease, 39% of those treated were able to be maintained at the target Cp for this duration.

The pattern and speed of progression observed in these patients have also provided useful information. One patient achieved stable disease at all sites but one, and has chosen to remain on TM therapy due to disease stabilization at the more life-threatening sites of disease (bowel and paratracheal lymph nodes). Interestingly, the site of progression in this patient with melanoma is a large adrenal metastasis, which is at present being irradiated. This and other observations in this trial suggest that, whereas copper deficiency may be generally inhibitory of angiogenesis, heterogeneity of tumor type and the specific location of metastases may modulate the response to this therapeutic modality. Since it appears that lesions progress at a much faster rate upon copper repletion than while on TM therapy, adjunct modalities, either systemically or local-regionally, may be used to address the specific sites of progression, while allowing the patients to remain in a copper deficient state.

These studies also show that combination therapies of TM with radiotherapy, trastuzumab, and interferon-alpha, occur without apparent exacerbation of toxicity of the added modality. Taken as a whole, the safety and preliminary efficacy data derived from this trial supports the use of TM, TP-TM and related compounds alone, or in combination, for the

treatment of early metastatic disease, minimal disease, and in adjuvant high-risk clinical settings, including chemoprevention.

EXAMPLE 4

Phase II/III Clinical Trial of TM as Anti-Cancer Therapy

A. Introduction

Four considerations enter into designing the drug dose and schedule for this trial. The first is that the dose regimens previously used, although effective in reducing copper, take too long to get to the Cp endpoint in order to properly evaluate efficacy. As it is known that tumors sequester copper (Apelgot *et al.*, 1986; Arnold and Sasse, 1961), anti-angiogenic effects will not likely be detected until systemic copper deficiency has been maintained perhaps for at least a few weeks to months. Thus, it is important to get to the endpoint of systemic copper deficiency (0-20% Cp level) as quickly as possible to maximize the potential for efficacy. Thus, the present trial utilizes a "loading" dose, to be used for two weeks, to achieve Cp criteria, followed by a lower maintenance dose, to remain at the target Cp of 0-20% baseline. This design utilizes the knowledge gained in the Phase I trial and will determine the efficacy of TM, TP-TM and related compounds to provide stable disease or tumor decrease.

Second, the trial will evaluate whether an efficacious response depends on how rigorously Cp levels are controlled. Thus, in the first group of patients, Cp levels are maintained between 10 and 20%, and in the second group Cp levels are maintained between 0 and 10%.

Third, this trial will determine whether maintenance of a low copper status is more easily maintained with zinc therapy. The inventors have developed zinc as an FDA approved therapy for Wilson's disease. It acts by inducing intestinal metallothionein and blocking the absorption of copper. Thus in 6 patients, the low copper status will be maintained by using 25 mg of zinc tid, with dose adjustments as necessary to maintain Cp criteria.

B. Phase II Study

1. Loading Dose

The inventors have found that a dosing schedule of 20 mg tid with meals, and a single dose of 60 mg between meals reduces the Cp level to <20% more rapidly than 20 mg tid with meals and 20 mg tid between meals. Therefore, this dosing schedule will be studied in a Phase II study. Three other loading doses of TM will also be studied: Level 1: 20 mg tid between meals and 20 mg tid with meals until Cp<20% (10 patients); Level 2: 25 mg tid between meals and 25 mg tid with meals until Cp<20% (10 patients); and Level 3: 30 mg tid between meals and 30 mg tid with meals until Cp<20% (10 patients).

The objective of the loading dose study is to arrive at the desired Cp level (<20% of baseline) within 2-3 weeks. The trial will begin with Level 1. If Level 1 achieves the desired Cp level, all 30 patients will be loaded at dose Level 1. If Level 1 does not achieve the desired Cp level, the trial will move to loading dose Level 2, and on to dose Level 3 if necessary. Each of these doses are safe for weeks to a few months.

2. Maintenance Dose of TM

Two levels of maintenance dose will be studied: Level 1: TM doses adjusted as necessary to maintain Cp at 10-20% of baseline (12 patients); and Level 2: TM doses adjusted as necessary to maintain Cp at 0-10% of baseline (12 patients).

The objective of comparing two maintenance dose levels is to get a general picture of whether more stringent copper control tends to enhance efficacy. In general, the tumor types will be randomized between the two levels.

3. Maintenance Dose of Zinc

Two patients from each loading dose group, for a total of six patients, will be treated with zinc therapy for maintenance control. The trial will begin with 25 mg of zinc 3 times per day (away from food) and adjust the dose to maintain Cp below 20% of baseline.

The objective of the zinc study is to see if it is easier to control copper status during maintenance with zinc than with TM, and to get a general picture of whether efficacy with zinc is generally comparable to efficacy with TM.

4. Patient Selection Criteria

The patient selection criteria will include: a) metastatic adenocarcinoma, squamous carcinoma, or sarcoma of any organ of origin; b) measurable disease by chest X-ray, CAT Scan, or plain bone films; c) progressive disease documented at least once within 3 months of entry; d) ability to provide informed consent; e) performance status ECOG 0-1; and f) life expectancy $\geq$ 6 months.

Exclusion criteria include hematocrit less than 29, LFT's more than four times normal, or severe concurrent medical disease requiring intensive management.

5. Parameters

The parameters that will be followed in the patients are: 1) CBC platelets, weekly; 2) electrolytes, BUN, creatinine, LFT's weekly; 3) blood for serum copper, molybdenum, ceruloplasmin weekly; 4) urinalysis weekly; 5) clinical status every 2 weeks; 6) tumor measurement every 4 weeks; and 7) research angiogenesis – sensitive ultrasound every 8 weeks.

6. Toxicity Endpoints

As before, the drug will be stopped when either hematocrit or WBC drops below 80% of baseline. The drug will also be stopped if there is evidence of possible systemic toxicity, such as abnormal liver or renal function tests, or any other grade 3 or higher toxicity by NCI criteria.

7. Length of Study

If there is not evidence of disease stabilization or reduction in a patient by 6 months, the study in that patient will be terminated. Otherwise, therapy will continue as long as the disease is controlled within 25% of the initial size at all sites, there is not significant toxicity, and the patient desires to continue.

EXAMPLE 5

Tetrapropylammonium Tetrathiomolybdate (TP-TM)

A. Preparation

(NH4)2[MoS4] (5.2 g, 20 mmol) was dissolved in water (100 ml) and the filtrate solution was added to 1.0M of nPr4NOH water solution (40 ml). The color of solution changed to bright red and the reaction mixture formed a red precipitate. After stirring for an hour, the precipitate was filtered off and air-dried. The filtrate was removed water by N2 streaming, which produced more products. Usually 10-11 g (84-92% yield) of crystalline product was isolated. The compound was characterized by XRD, FT-IR, UV-VIS and CHN analysis. The CHN analyses of every preparation showed a little low C and N contents, which may be due to the water, but XRD measurement ensured that all the compounds had the same crystal system.

B. X-ray crystallography

The needle shaped crystal obtained from concentrated water solution was isolated and diffraction data were collected on a CCD diffractometer. The compound crystallizes in the monoclinic system. Unit cell: a = 32.067(3)Å b = 13.7236(15)Å c = 14.8379(16)Å and $\beta = 109.241(2)°$. The structure was solved in the space group, C2/C. The crystal packing projection of (NH4)2[MoS4] is shown in FIG. 1.

The X-ray powder pattern calculated on the basis of atomic coordinates obtained from the single-crystal X-ray structure determination is shown in FIG. 2, and the X-ray powder pattern obtained experimentally from the crystalline $(n\text{-}Pr_4N)_2MoS_4$ is shown in FIG. 3. It can be concluded that the crystalline material is identical to the single crystal used for structure determination.

Table 3

Crystal Data and Structure Refinement for (^{n}Pr$_4$N)[MoS$_4$]

Identification Code	**ProMoS4**
Empirical formula	C24 H56 Mo N2 S4
Formula weight	596.89
Temperature	158(2) K
Wavelength	0.71073 A
Crystal system, space group	Monoclinic, C2/c
Unit cell dimensions	a = 32.067(3) A alpha = 90 deg.
	b = 13.7236(15) A beta = 109.241(2) deg.
	c = 14.8379(16) A gamma = 90 deg.
Volume	6165.1(12) A^3
Z, Calculated density	8, 1.286 Mg/m^3
Absorption coefficient	0.711 mm^-1
F(000)	2560
Crystal size	0.24 x 0.06 x 0.04 mm
Theta range for data collection	1.35 to 26.45 deg.
Limiting indices	-40<=h<=40, -17<=k<=17, -18<=1<=18
Reflections collected/unique	30260 / 6343 [R(int) = 0.0983]
Completeness to theta = 26.45	99.8%
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	6343 / 0 / 281
Goodness-of-fit on F^2	1.138
Final R indices [I>2sigma(I)]	R1 = 0.0780, wR2 = 0.1483
R indices (all data)	R1 = 0.1056, wR2 = 0.1560
Largest diff. peak and hole	1.179 and –1.481 e.A^-3

5

Table 4

Atomic coordinates (×10^4) and equivalent isotropic displacement parameters (A^2 × 10^3) for (^{n}Pr$_4$N) [MoS$_4$] U(eq) is defined as one third of the trace of the orthogonalized Uij tensor

	X	y	z	U(eq)
Mo(1)	1271(1)	2441(1)	868(1)	19(1)
S(1)	800(1)	1687(1)	1416(1)	24(1)
S(2)	1498(1)	1435(2)	-8(1)	32(1)
S(3)	1844(1)	2946(1)	2047(1)	27(1)
S(4)	947(1)	3686(1)	-10(1)	29(1)
N(1)	2461(2)	-120(4)	-1007(4)	17(1)
N(2)	0	4123(6)	2500	18(2)
N(3)	0	9204(6)	2500	26(2)
C(1)	2519(2)	327(5)	-30(5)	19(1)
C(2)	2808(2)	1222(5)	230(4)	21(1)
C(3)	2816(2)	1584(5)	1218(4)	24(2)
C(4)	2903(2)	-380(5)	-1107(4)	19(1)
C(5)	3190(2)	-1056(6)	-342(5)	27(2)
C(6)	3604(2)	-1309(6)	-568(5)	31(2)
C(7)	2175(2)	-1030(5)	-1114(5)	19(1)
C(8)	1688(2)	-856(5)	-1293(5)	22(1)
C(9)	1474(2)	-1796(5)	-1119(5)	22(1)
C(10)	2238(2)	628(5)	-1777(4)	21(1)
C(11)	2148(2)	267(6)	-2795(5)	29(2)
C(12)	1889(3)	1040(7)	-3483(5)	49(2)
C(13)	398(2)	4774(5)	2639(4)	20(1)
C(14)	832(2)	4255(6)	2755(6)	31(2)
C(15)	1180(2)	4996(6)	2761(6)	36(2)
C(16)	71(2)	3459(5)	3362(4)	17(1)
C(17)	107(2)	3986(5)	4289(4)	24(2)
C(18)	198(2)	3235(5)	5094(5)	25(2)
C(19)	362(3)	8599(13)	2441(12)	170(10)
C(20)	693(5)	8281(16)	2611(9)	206(13)
C(21)	1041(2)	7746(7)	2560(6)	45(2)
C(22)	192(4)	9816(12)	3356(8)	139(8)
C(23)	459(3)	10043(14)	3997(14)	214(14)
C(24)	603(3)	10634(7)	4818(6)	44(2)

Table 5

Bond lengths [A] and angles [deg] for ($^{n}Pr_4N$)[MoS_4]

Mo(1)-S(2)	2.1780(19)
Mo(1)-S(3)	2.1906(18)
Mo(1)-S(4)	2.1921(18)
Mo(1)-S(1)	2.1957(18)
N(1)-C(4)	1.517(7)
N(1)-C(7)	1.525(8)
N(1)-C(1)	1.529(8)
N(1)-C(10)	1.527(8)
N(2)-C(13)	1.516(7)
N(2)-C(13)#1	1.516(7)
N(2)-C(16)	1.525(7)
N(2)-C(16)#1	1.525(7)
N(3)-C(19)	1.454(11)
N(3)-C(19)#1	1.454(11)
N(3)-C(22)	1.477(11)
N(3)-C(22)#1	1.477(11)
C(1)-C(2)	1.511(9)
C(2)-C(3)	1.539(9)
C(4)-C(5)	1.519(9)
C(5)-C(6)	1.512(9)
C(7)-C(8)	1.515(8)
C(8)-C(9)	1.523(9)
C(10)-C(11)	1.524(9)
C(11)-C(12)	1.516(10)
C(13)-C(14)	1.520(9)
C(14)-C(15)	1.507(9)
C(16)-C(17)	1.526(9)
C(17)-C(18)	1.531(9)
C(19)-C(20)	1.097(13)
C(20)-C(21)	1.357(12)
C(22)-C(23)	1.095(14)
C(23)-C(24)	1.410(13)
S(2)-Mo(1)-S(3)	108.67(7)
S(2)-Mo(1)-S(4)	109.01(8)
S(3)-Mo(1)-S(4)	109.64(8)
S(2)-Mo(1)-S(1)	109.18(8)
S(3)-Mo(1)-S(1)	110.57(7)
S(4)-Mo(1)-S(1)	109.75(7)
C(4)-N(1)-C(7)	110.3(5)
C(4)-N(1)-C(1)	111.2(5)
C(7)-N(1)-C(1)	108.0(5)
C(4)-N(1)-C(10)	107.9(5)
C(7)-N(1)-C(10)	110.9(5)
C(1)-N(1)-C(10)	108.6(5)
C(13)-N(2)-C(13)#1	107.8(7)

C(13)-N(2)-C(16)	110.5(3)
C(13)#1-N(2)-C(16)	110.7(3)
C(13)-N(2)-C(16)#1	110.7(3)
C(13)#1-N(2)-C(16)#1	110.5(3)
C(16)-N(2)-C(16)#1	106.6(7)
C(19)-N(3)-C(19)#1	110.3(16)
C(19)-N(3)-C(22)	105.2(6)
C(19)#1-N(3)-C(22)	112.8(11)
C(19)-N(3)-C(22)#1	112.8(11)
C(19)#1-N(3)-C(22)#1	105.2(6)
C(22)-N(3)-C(22)#1	110.7(14)
C(2)-C(1)-N(1)	116.2(5)
C(1)-C(2)-C(3)	108.8(5)
N(1)-C(4)-C(5)	115.2(5)
C(6)-C(5)-C(4)	109.4(5)
C(8)-C(7)-N(1)	116.0(5)
C(7)-C(8)-C(9)	109.5(5)
C(11)-C(10)-N(1)	114.3(5)
C(12)-C(11)-C(10)	108.9(6)
N(2)-C(13)-C(14)	116.0(6)
C(15)-C(14)-C(13)	109.5(6)
N(2)-C(16)-C(17)	114.8(5)
C(16)-C(17)-C(18)	108.8(6)
C(20)-C(19)-N(3)	160.8(12)
C(19)-C(20)-C(21)	162.1(13)
C(23)-C(22)-N(3)	153.3(11)
C(22)-C(23)-C(24)	148.8(12)

Symmetry transformations used to generate equivalent atoms: #1 –x,y,-z+1/2

Table 6

Anisotropic displacement parameters (A^2 × 10^3) for (^{n}Pr$_4$N)[MoS$_4$]
The anisotropic displacement factor exponent takes the form:
-2 pi^2 [h^2 a*^2 U11 + ... + 2 h k a* b* U12]

	U11	U22	U33	U23	U13	U12
Mo(1)	18(1)	23(1)	16(1)	5(1)	7(1)	5(1)
S(1)	22(1)	29(1)	23(1)	4(1)	9(1)	1(1)
S(2)	31(1)	39(1)	28(1)	-2(1)	13(1)	14(1)
S(3)	23(1)	34(1)	21(1)	7(1)	5(1)	-4(1)
S(4)	27(1)	32(1)	28(1)	13(1)	11(1)	10(1)
N(1)	18(3)	20(3)	13(3)	-2(2)	7(2)	1(2)
N(2)	19(4)	21(4)	17(4)	0	8(3)	0
N(3)	32(5)	22(5)	31(5)	0	19(4)	0
C(1)	21(3)	23(3)	13(3)	-3(3)	7(2)	-4(3)
C(2)	20(3)	27(4)	15(3)	2(3)	5(3)	-2(3)
C(3)	24(3)	28(4)	17(3)	-8(3)	5(3)	0(3)
C(4)	19(3)	22(4)	18(3)	-2(3)	10(3)	4(3)
C(5)	23(3)	33(4)	27(4)	9(3)	13(3)	10(3)
C(6)	26(4)	30(4)	42(5)	6(4)	20(3)	7(3)
C(7)	25(3)	16(3)	16(3)	-1(3)	5(3)	0(3)
C(8)	23(3)	23(4)	16(3)	-2(3)	3(3)	0(3)
C(9)	23(3)	24(4)	19(3)	-5(3)	7(3)	-1(3)
C(10)	23(3)	26(4)	14(3)	5(3)	7(3)	4(3)
C(11)	35(4)	36(4)	13(3)	1(3)	5(3)	5(3)
C(12)	71(6)	57(6)	14(4)	7(4)	6(4)	32(5)
C(13)	30(4)	21(4)	11(3)	-6(3)	7(3)	-10(3)
C(14)	21(3)	34(4)	41(4)	-8(4)	14(3)	-10(3)
C(15)	34(4)	43(5)	34(4)	-8(4)	16(3)	-13(4)
C(16)	19(3)	20(3)	13(3)	2(3)	5(2)	0(3)
C(17)	33(4)	25(4)	14(3)	1(3)	9(3)	-2(3)
C(18)	21(3)	36(4)	18(3)	3(3)	6(3)	-1(3)
C(19)	20(5)	219(18)	236(18)	-206(16)	-6(7)	16(8)
C(20)	121(11)	350(30)	78(9)	-127(13)	-65(8)	184(16)
C(21)	32(4)	66(7)	34(4)	-18(4)	6(3)	7(4)
C(22)	103(10)	212(17)	52(7)	-89(9)	-41(7)	119(11)
C(23)	24(5)	270(20)	280(20)	-250(20)	-44(9)	46(9)
C(24)	58(5)	45(5)	38(5)	-9(4)	26(4)	-10(4)

Table 7

Hydrogen coordinates (× 10^4) and isotropic displacement parameters (A^2 × 10^3) for ($^{n}Pr_4N$)[MoS_4]

	x	y	z	U(eq)
H(1A)	2223	499	-5	22
H(1B)	2644	-176	463	22
H(2B)	2691	1738	-253	25
H(2C)	3112	1061	247	25
H(3B)	3002	2167	1392	35
H(3C)	2937	1073	1693	35
H(3D)	2515	1743	1196	35
H(4A)	3070	230	-1098	23
H(4B)	2850	-690	-1737	23
H(5A)	3270	-734	290	32
H(5B)	3024	-1658	-316	32
H(6A)	3789	-1745	-74	46
H(6B)	3768	-711	-585	46
H(6C)	3523	-1633	-1190	46
H(7A)	2206	-1422	-1649	23
H(7B)	2293	-1424	-526	23
H(8A)	1544	-637	-1960	26
H(8B)	1650	-339	-861	26
H(9A)	1158	-1684	-1237	33
H(9B)	1615	-2004	-456	33
H(9C)	1511	-2304	-1550	33
H(10A)	1955	826	-1701	25
H(10B)	2428	1214	-1676	25
H(11A)	2430	137	-2908	35
H(11B)	1976	-347	-2895	35
H(12A)	1828	814	-4140	74
H(12B)	2061	1643	-3384	74
H(12C)	1609	1161	-3370	74
H(13A)	438	5181	3212	25
H(13B)	334	5218	2084	25
H(14A)	926	3883	3360	37
H(14B)	792	3792	2222	37
H(15A)	1460	4662	2840	54
H(15B)	1219	5452	3291	54
H(15C)	1088	5355	2157	54
H(16A)	345	3080	3460	21
H(16B)	-177	2991	3217	21
H(17A)	-172	4336	4221	29
H(17B)	349	4469	4440	29
H(18A)	220	3566	5693	37
H(18B)	476	2897	5163	37
H(18C)	-44	2760	4941	37
H(19A)	204	7971	2391	204
H(19B)	326	8759	1769	204

H(20A)	716	8067	3263	247
H(20B)	852	8912	2722	247
H(21A)	1306	7920	3092	68
H(21B)	1090	7880	1955	68
H(21C)	977	7051	2597	68
H(22A)	121	10462	3045	167
H(22B)	-41	9718	3651	167
H(23A)	687	10208	3706	257
H(23B)	551	9397	4293	257
H(24A)	915	10497	5167	67
H(24B)	426	10495	5230	67
H(24C)	570	11322	4630	67

C. Stability

Four different samples were checked over the time. Dried crystals were kept in the closed bottle and open bottle. Ground crystals, dry and wet, were kept in the open bottle. UV-Visible spectroscope was used for its stability and 1 mg of compound was dissolved in 20 ml of water to prepare the solution. The compound was stable over 2 months period and the change of absorbance was within the experimental error range.

EXAMPLE 6

Enhanced Stability of TP-TM

Following the initial indication of enhanced stability, a study was done to compare the stability of TP-TM versus the original TM preparation (AmmTM). This study was done under conditions that exacerbate instability, *i.e.* the drugs were in open Petri dishes at room temperature. The differences in the activity of the two drugs over time are shown in Table 8 and in FIG. 4.

Table 8

Improved Stability of TP-TM

Day	TPTM	AmmTM
0	100	100
45	86	44
148	65	9
211	38	0

From these studies, the half life of TPTM under these conditions was surprisingly determined to be about 180 days. In contrast, the half life of the original TM (AmmTM) under the same conditions was determined to be about 40 days. This difference in stability makes TPTM a greatly improved drug over TM because it means it can be handled pharmaceutically in bulk without exquisite attention to air exclusion. It also means that the shelf life of the eventual formulation under similar conditions will be much better for TPTM.

EXAMPLE 7

Anti-Tumor Effects of TP-TM *In Vivo*

As TM has safe, but effective anti-angiogenic and anti-tumor actions in animals and humans, and as TP-TM was designed to maintain the important copper binding actions but to have increased stability, it was expected that TP-TM would also have advantageous anti-angiogenic and anti-tumor effects. The following study was designed to confirm these properties *in vivo*.

In this study, 10^6 breast cancer cells were injected in the mammary fat pad of 4 groups of athymic, nude mice (5 mice per group). Control mice received no treatment. The other three groups were treated with either the original TM (AmmTM, 1 mg/day) or with two different doses of TP-TM (1 mg/day and 1.5 mg/day), and the tumor volume over the course of treatment was measured.

As shown in FIG. 5, the growth curves of the treated animals are significantly different from the control animals ($p < 0.01$), with both TM and TP-TM effectively inhibiting tumor growth over the course of the study. Importantly, the TP-TM growth curves (labeled "TP" on FIG. 5) are statistically indistinguishable from the TM curves, showing that the new compound has equal biological efficacy in inhibiting *in vivo* tumor growth as the parent compound. In addition, this study shows that a dose of 1 to 1.5 mg/mouse/day of TP-TM is sufficient for efficacy.

EXAMPLE 8

Safety of TP-TM *In Vivo*

The data in Examples 6 and 7 show that TP-TM has increased stability over the parent compound, TM, but that it has essentially the same anti-tumor effects in controlled studies *in vivo*. The present example shows that the salt released from TP-TM is non-toxic in mice at doses many higher than those required for clinical treatment.

To confirm the non-toxic nature of the breakdown product of TP-TM, tetrapropyl ammonium chloride was administered at doses of 2, 20, 40, 60, 80, 100, and 200 mg/mouse/day to groups of 3-4 mice. For doses of 2 and 20 mg/mouse/day, all animals were alive and healthy after 58 doses (Table 9).

Table 9

Tetrapropyl Ammonium Chloride Acute and Subacute Toxicity Study (Single daily dose by gavage in mice)

Dose per mouse (mg TPCI)	Mg TP/kg	Human Equiv/kg Loading dose (x as high)	Human Equiv/kg Maintenance dose (x as high)	Results
2	84	23.3	46.6	4 of 4 alive after 58 doses
20	840	233	466	4 of 4 alive after 58 doses
40	1,680	466	932	3 mice total: 1 died, 4 doses; 1 died, 27 doses; 1 died, 28 doses
60	2,520	699	1,398	3 mice total: 1 died, 3 doses; 2 died 4 doses
80	3,360	932	1,864	3 mice total: 2 died, 2 doses; 1 died, 3 doses
100	4,200	1,165	2,330	4 mice total: 1 died, 1 dose; 3 died 2 doses
200	8,400	2,333	4,666	4 died, 1 dose
	Typical dose	3.6 mg/kg	1.8 mg/kg	

.Table 9 also depicts the human equivalents of the tetrapropyl ammonium chloride doses, scaling to a larger mammal. There was no detectable toxicity in the mice using doses up to 20-fold higher than required for tumor inhibition in the study described above (Example

7). These dose levels represent 46.6- and 466-fold higher doses of tetrapropyl that a human would be exposed to, for an equivalent biological copper-reducing effect of TP-TM. These data indicate that the salt released from TP-TM is non-toxic in mice at doses many fold higher than could possibly be required for copper-reducing therapy with this compound in humans.

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods, and in the steps or in the sequence of steps of the methods described herein, without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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